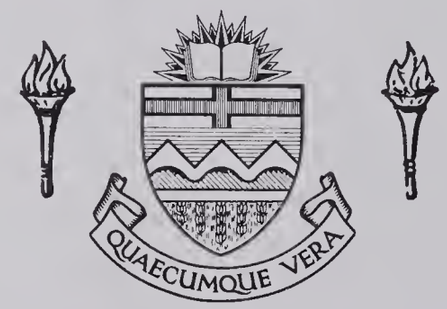

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THE EFFECT OF ACTIVATING AGENTS ON THE SPORES OF TWO BACILLUS SPECIES

BY

EDWARD J. NATH

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

DEPARTMENT OF FOOD SCIENCE

EDMONTON, ALBERTA

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FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance a thesis entitled

THE EFFECT OF ACTIVATING AGENTS
ON THE SPORES OF TWO BACILLUS SPECIES

submitted by Edward John Nath, in partial fulfillment of the requirements for the degree of Master of Science.

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ABBREVIATIONS USED IN THESIS

DPA	Dipicolinic acid
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
DAP	Diaminopimelic acid
RT	Room temperature
mRNA	messenger Ribonucleic acid
NAD	Nicotinamide-adenine dinucleotide

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ABSTRACT

The activation of spores of Bacillus subtilis 8057 and Bacillus cereus 127 by various treatments was investigated. The effect of heat, change in pH, the addition of reducing agents and cations were studied separately and in combination. Spore suspensions of B. subtilis 8057 exhibited heat-activation response throughout the entire range of treatment times (10 - 100 min). A temperature of 95°C for 10 min gave maximum activation, while 90°C treatment for 10 min resulted in heat-induced dormancy. The pH value of the spore suspensions had a marked effect on activation; pH 5.0 at 95°C gave maximum activation up to 5 min treatment time, after which it was lethal to the spores. A highly acid or alkaline pH with heat also resulted in lethal effects except for pH 8.0 at 70°C at 10 min, which gave activation equivalent to that of pH 7.0 at 95°C at 10 min. Thioglycolic acid activated the spores but this effect was lessened in the presence of heat. Cations (Ca^{++}, Mg^{++}, Mn^{++}) with heat produced a lethal effect, but the unheated spores were activated by the addition of the above cations, Mg^{++} giving the best results. B. cereus 127 gave no significant response to any of the above activating agents with the exception of heating at 65°C for 40 min.

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GENERAL INTRODUCTION

The process by which dormant bacterial spores change into fully active vegetative cells can be divided into three distinct phases: activation, germination and outgrowth (Keynan and Halvorson, 1965; Gibbs, 1967). Sussman (1965) defines activation as the application of external stimuli which induce germination. Conversely, it may be thought of as the process responsible for breaking dormancy.

Activation is a reversible process and if the activated spore is not induced to germinate it will revert to its dormant state (Curran and Evans, 1945; Powell, 1951; Keynan, Evenchik, Halvorson and Hastings, 1964; Keynan, Issahary-Brand and Evenchik, 1965; Keynan and Halvorson, 1965) and has been found capable of surviving in that state for as long as three hundred years (Sneath, 1962).

The conditions required to break dormancy vary greatly among the species of bacteria. The heating of spores in an aqueous suspension (Curran and Evans, 1945; Keynan and Halvorson, 1965) has been the most common method used. However, Hyatt, Homes and Levinson (1966) used water vapor as a means of activation, and aging alone has been found effective (Keynan, Issahary-Brand and Evenchik, 1965).

Krabbenhoft, Corlett and Anderson (1966) have shown interest in the use of irradiation to break dormancy, but in the

past decade most attention has been turned to the use of chemical agents (Keynan, Issahary-Brand and Evenchik, 1965; Gibbs, 1967) as a means of facilitating the activating process.

The purpose of the present investigation was to study several aspects of the activation of spores of Bacillus subtilis 8057 and Bacillus cereus 127. Among these were:

(i) the minimal time-temperature treatment which will produce maximum activation,

(ii) the effect of pH on activation,

(iii) the effect of cations on activation, and

(iv) the effect of reducing agents on activation.

THE NATURE OF SPORES

STRUCTURE

The formation of spores is accompanied by many morphological and biochemical changes, which, in recent times, have been carefully elucidated by researchers in this field. In Fig. 1, Halvorson (1965) has co-ordinated the nuclear and structural changes in sporulation (Young and Fitz-James, 1959a) with changes in metabolic activity (Halvorson and Srinivasan, 1964) and the initiation of the biosynthesis of cellular components (Vinter, 1962; Halvorson, 1965). The time scale is 6 - 8 hr. within each time interval, and the order of events is arbitrary.

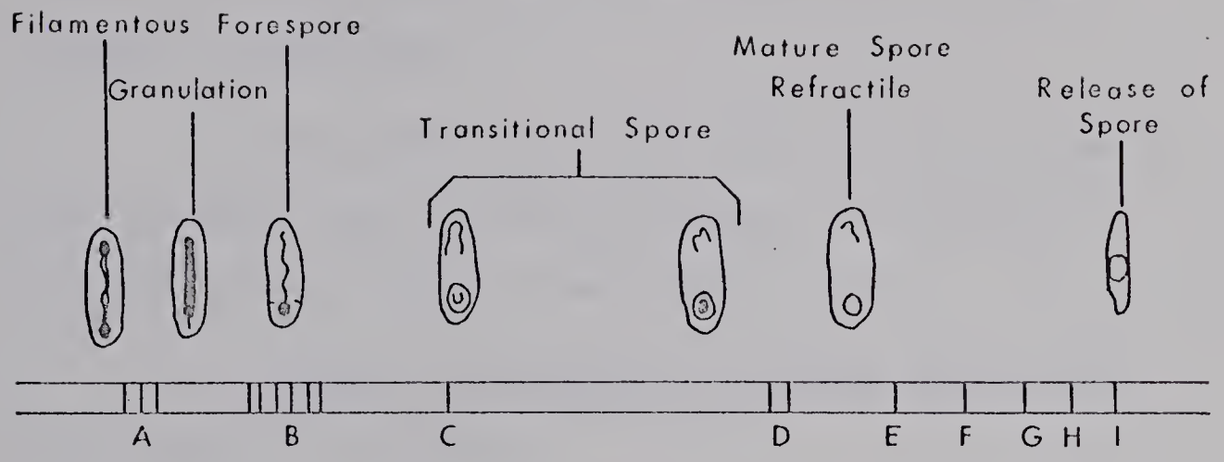

Fig. 1. Sequential appearance of morphological and biochemical components during sporulation in Bacillus species. (Diagram modified from that of Halvorson, 1965).

A. This stage indicates the commitment to sporulation, an irreversible stage at which even if the stimulation is removed, sporulation continues unabated. This stage is characterized by the production of a sporulation factor, protease, and an antibiotic. The sporulation factor appears at the time of granulation, and initiates sporulation (Sussman and Halvorson, 1966).

B. Acetate oxidation involves the TCA and glyoxylic acid cycle enzymes such as glucose dehydrogenase, adenosine deaminase, spore catalase, and aceto-acetyl CoA reductase. These enzymes appear at the time of maximal cell growth (Bach and Sadoff, 1962).

C. Ribosidase appears at this stage.

D. S-S proteins are formed and the spore becomes resistant to irradiation.

E. The synthesis of dipicolinic acid becomes apparent about two hours after the period of maximal cell growth; at this time the spore begins to incorporate calcium.

F. Following the formation of DPA-Ca chelates, the spore becomes heat-resistant.

G. Alanine racemase is synthesized in the spore about 4 hr. after the period of maximal growth. This enzyme is heat-resistant and little of it is found in the vegetative cells (Stewart and Halvorson, 1953). It is probably associated with the outer spore coat or exosporium.

H. A lytic system is produced.

I. The spores are released from the host cell.

Graphically, the stages of spore formation are represented in Fig. 2.

During sporulation there appears at one end of the bacterial cell, a forespore, which encloses part of the cytoplasm, and half of the cellular DNA (Young and Fitz-James, 1959), which gradually matures into a dormant endospore, composed of complex internal structures quite different from those of the vegetative cell. Spores and vegetative cells differ in other ways as well:

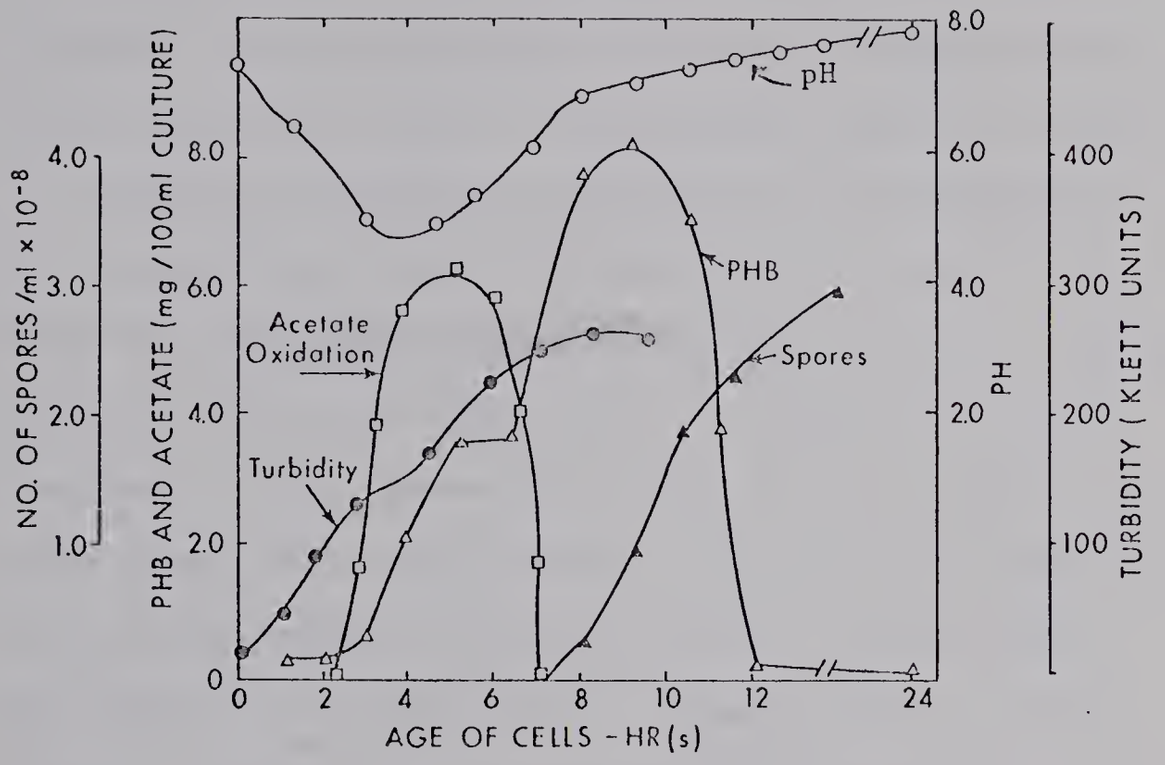

Fig. 2. Changes in the pH, turbidity, and poly-β (OH) butyric acid concentration during growth and sporulation of Bacillus cereus T (from Kominek and Halvorson, 1965). Although acetate oxidation is not shown in the original graph, it is indicated in the text and is added here.

(i) Chemical composition. Spores have 5 - 15% of their dry weight as dipicolinic acid (DPA), a strong chelating agent, which is not found in the vegetative cells. Since DPA is released upon germination and the spore cortex disappears at the same time, it has been proposed that the spore DPA is located in some combination within the cortex (Mayall and Robinow, 1957; Rode and Foster, 1960a and b). It appears that several complexes may exist between DPA-calcium and the proteins of the spore (Young, 1959; Berger and Marr, 1960; Church and Halvorson, 1959; Black, Hashimoto and Gerhardt, 1960). It seems likely that DPA is synthesized within the forespore since it appears after the formation of this structure (Halvorson, 1957), and is neither liberated nor found associated with the vegetative cell protein, or the spore debris fraction during lysis of the sporulating cell (Halvorson and Howitt, 1961). These workers suggest that DPA within the spore may have an indirect influence on heat resistance, and may be responsible for such functions as calcium accumulation, stimulation of electron transport, enzyme stability, dormancy, and the stimulation of germination under the proper conditions.

Spores differ from vegetative cells also in that they are enriched in divalent metals and S-S sulfur, but contain little water, and only half as much DNA as the vegetative cells (Halvorson, 1965).

The spore walls of Bacillus subtilis are largely composed of structural protein whereas the vegetative cell walls are a peptide-amino acid-sugar complex (Salton and Marshall, 1959). The high nitrogen content of the spore walls is especially evident in Table 1.

Sporulation depends on the utilization of the free amino acid pools, and parallels the degradation of available purines and pyrimidines (Foster and Perry, 1954; Widra, 1956; Young and Fitz-James, 1959b; Murrell, 1961; Halvorson, 1962). Vinter (1960a, b) stated that the main component of this new spore protein is cystine. He noted that spores contain two to five times as much cystine (plus cysteine) as vegetative cells. Using ^{35}S-cysteine, Vinter (1960) showed that the new protein

Table 1. Analysis of spore walls of vegetative cells of Bacillus subtilis (Salton and Marshall, 1959).

Constituents as % total dry weight

Constituent	Veg. Cell	Spore
	Dry Weight %	
Total N	4.6	12.9
Total P	4.2	1.4
Hexosamine	10.7	2.2
Glucosamine	7.9	1.5
Muramic acid	2.3	0.6
DAPA	5.6	-
Lipid	0.7	3.0
O-ester groups (as O-acetyl)	0.255	0.255

is formed during the "young spore" stage, and is incorporated into the insoluble part of the spore coat, a process which occurs prior to the development of the heat resistance of the spore. Table 2 shows the amino acid composition of the cell walls of vegetative cells and spores of Bacillus subtilis.

Table 2. The amino acid composition of cell walls of Bacillus subtilis (Salton, 1960).

(Relative Molecular Proportions)

Amino Acid	Veg. Cell	Spore
Lysine	0	6
Glycine	0	5
Serine	Trace	4
Alanine	4	4
Glutamic acid	2	3
Valine/methionine	Trace	2
Leucine/isoleucine	Trace	2
Tyrosine	0	1.5
Aspartic acid	0	1
DAPA	1	1
Threonine	0	1

(ii) Metabolic patterns. Differences in the enzymatic content of spores and vegetative cells have been reported (Simmons and Costilow, 1962), and the activities of some of these enzymes increase within the spore. Moreover, the method of electron transport would seem to differ in spores since the oxidative particles used in the vegetative cells are either lost or made incomplete during sporulation (Doi and Halvorson, 1961).

Bennett (1956) has suggested that membrane movement and vesiculation within the cell might provide a mechanism for active transport and ion pumping. The main pathway for electron transport in vegetative cells is a cytochrome system, while spores use a soluble flavo-protein oxidase for terminal oxidation (Sussman and Halvorson, 1966). Spores and vegetative cells differ in their cytochrome content (Keilin and Hartree, 1949), and are cyanide-sensitive (Spencer and Powell, 1952; Hachisuka, Asano, Kaneko and Kanbe, 1956). Spores contain <6% of the cytochrome found in vegetative cells, but the amount increases rapidly with germination (Nakata, 1957). Sussman and Halvorson (1966) concluded that spores contain altered or immature electron transport particles.

It has also been found that spores of Bacillus species have four times the soluble $NADH_2$ oxidase found in the vegetative cells (Szulmajster and Schaeffer, 1961). This plus the absence of certain oxidative particles (Doi and Halvorson, 1961) contributes to the dependence of spores on an electron transport system mediated by $NADH_2$ oxidation (Sussman and Halvorson, 1966).

The method of glucose metabolism in spores is changed as well, since both a functional TCA cycle and glyoxylic acid cycle appear (Halvorson, 1965) with the onset of sporulation. Blumenthal (1965)however, qualified this latter statement, saying that the

nature of the medium in which spores are placed will affect the subsequent development of the TCA cycle. Goldman and Blumenthal (1960, 1961, 1963, 1964) stated that whereas vegetative cells used the glyoxylic pathway almost exclusively, spores metabolized only 75% of their glucose in this way.

(iii) New species of macromolecules. Sporulation leads to the production of spore-specific antigens. In Bacillus cereus, the thermolabile antigens of the vegetative cell disappear, and eight new antigens are formed (Baillie and Norris, 1964). In addition, distinct and different proteins are formed in the spore which have enzymatic activities similar to the corresponding proteins in the vegetative cell (Lawrence and Halvorson, 1954).

Much additional information has become available about the interior of the bacterial spore, by the use of the electron microscope. Some species such as Bacillus cereus exhibit an exosporium (Gerhardt, Bannar and Ribi, 1961) which usually appears as a loose delicate envelope, and which may exhibit surface folds or ridges as in Bacillus polymyxa (Holbert, 1960), or a nap as in Bacillus anthracis (Gerhardt and Ribi, 1964). The exosporium of Bacillus anthracis was found to have a crystalline structure (Gerhardt and Ribi, 1964), and Hannay (1956) working with Bacillus thuringiensis, found that the exosporium was formed within the bacterial cell at the time of sporulation.

Beneath the exosporium lies a layer or layers, called

the spore coat, which gives the spore its characteristic shape. Bradley and Williams (1957) have shown that the surfaces of spores have different patterns; they may be dimpled, or contain grooves and ridges. The observations of Ellar and Lundgren (1966) seem to indicate that the combination of membrane fold and vesicular units in spores serves to localize synthetic activity at a particular site within the cell. Bradley and Franklin (1958) describe the elaborate contours which form the outer spore coat of Bacillus subtilis as being longitudinally ribbed with occasional transverse ribs at the ends. The ribbing was rather irregular, and it varied with the strain. The spore coat may be composed of several layers, each of which has a laminated structure (Holbert, 1960; Smith and Ellner, 1957; Tokuyasu and Yamada, 1959; Ohye and Murrell, 1962).

The spore coat is separated from the cortex by a thin membrane (Fitz-James, 1962a). Warth, Ohye and Murrell (1962, 1963) have indicated that the two coats surrounding the spore are largely protein, and the underlying cortex is mainly composed of a mucopeptide polymer similar to that found in the vegetative cell walls of the Gram-positive species. Although the exact mucopeptide structure is as yet unknown, Murrell and Warth (1965) have proposed a hypothetical structure which appears in Fig. 3. The cortex appears as a layered matrix, and is probably cross-linked, perhaps with chains twisted and coiled, or interwoven as they are laid down during cortical formation. It has been noted that the cortex is the

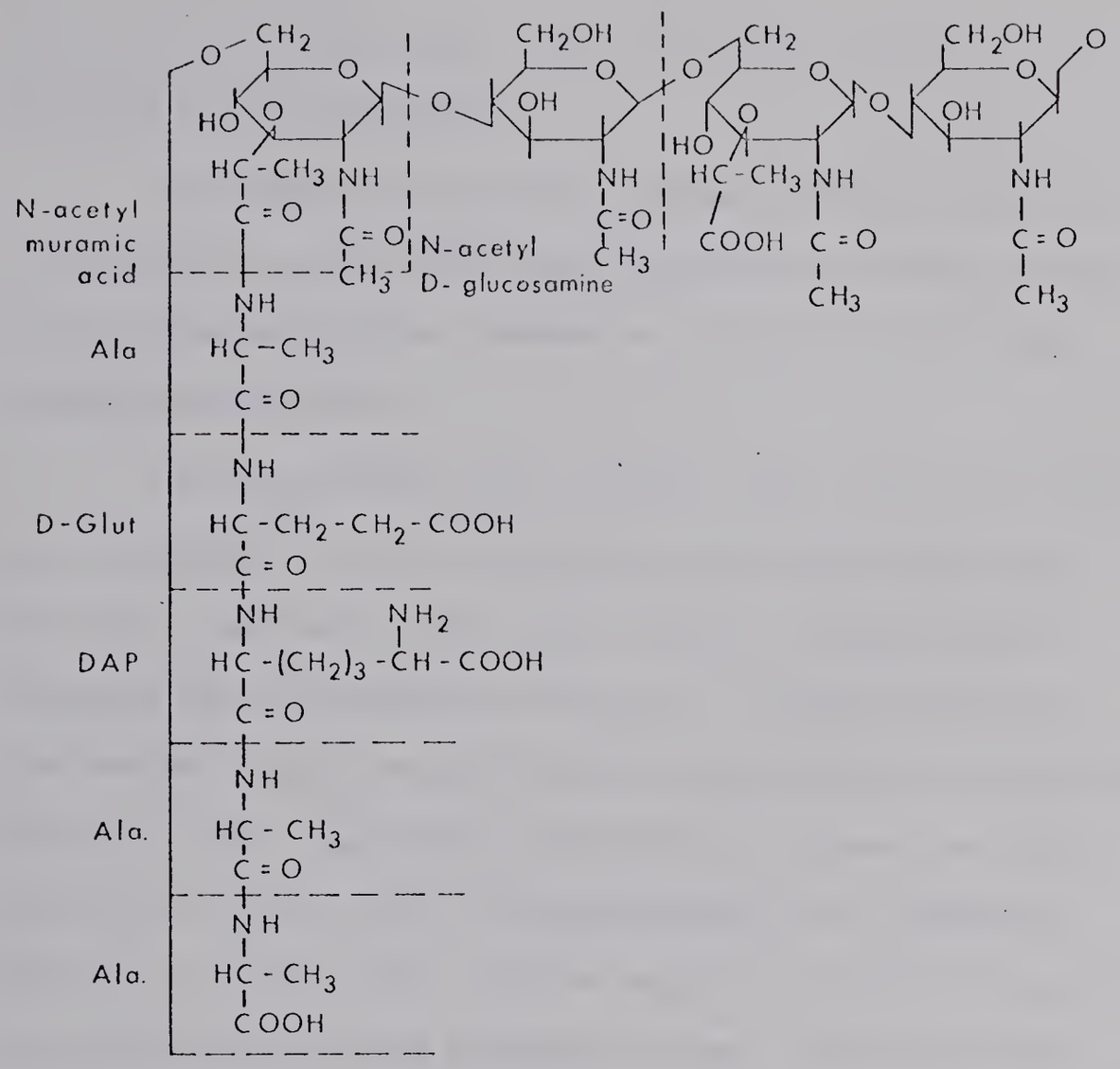

Fig. 3. Hypothetical structure of mucopeptide (Murrell and Warth, 1965).

proposed site (Mayall et al., 1957) of DPA which may have some influence on heat resistance.

The dormant cytoplasm is separated from the cortex by a thin membrane called a spore wall (Hashimoto and Naylor, 1958) which, during germination, becomes the cell wall of the newly formed vegetative cell.

Most researchers favor a theory which regards the cortex as a permeability barrier surrounding an anhydrous spore coat. Rode, Lewis and Foster (1962) felt that such a barrier was responsible for the dormancy of the spore. The early prevailing view was that the spores were dry and impermeable to water (Fischer, 1877), but recent experiments have shown that glucose and lipids are absorbed by the spores of Bacillus cereus var. terminalis (Gerhardt and Black, 1960) and this suggests that the cortex is not as impermeable as was previously thought. They also found increased permeability in heat killed (DPA free) spores which suggests that the DPA-Ca chelate plays a part in controlling spore permeability.

It is generally agreed that spores have a low water content (Sussman and Halvorson, 1966) and many attempts have been made to measure this. The view proposed by Lewith (1890) that a part of the spore is in an anhydrous state has been recently considered as one of the most likely explanations of the properties of the spore (Powell and Strange, 1953; Fitz-James and Vance, 1955;

Rode and Foster, 1960; Black, 1960; Lewis, Snell and Burr, 1960). Gerhardt and Black (1960) estimated that glucose permeates 40% of the weight or 51% of the volume of spores of Bacillus cereus, apparently by free diffusion since its uptake was relatively independent of environmental variables. Molecules of molecular weights up to 160,000 were able to permeate this spore. Compounds of molecular weights from 400 to 160,000 were taken up in inverse proportion to their molecular weights. From these data it is estimated that the surface of the spore contained pores varying in diameter from 100 to 200 Å.

An interesting question is how an anhydrous region of a spore can be created in an aqueous environment. Lewis, Snell and Alderton (1965) have tried to explain this by comparing the spore to a bud protected by specialized layers, one of the functions of which is to exert pressure on the core, and by reducing the water content of the core, to confer metabolic dormancy and resistance to heat and other agents.

ENZYMES AS TRIGGERS OF ACTIVATION

It has already been noted that the biosynthesis of enzymes within the young spore plays an important role in the sporulation process (Halvorson, 1965), and it seems likely that they also form a vital part of the activation mechanism of the spore.

The majority of the enzymes present in bacterial spores are inactive *in vivo*, and are found to be heat-sensitive once they are activated, Sussman and Halvorson (1966) concluded from this that their resistance *in vivo* to heat activation treatment must be due to some generalized mechanism for heat stability rather than to an intrinsic property of the enzymes themselves. An exception is seen in Campbell's (1961) work with obligate thermophilic strains of *Bacillus stearothermophilus* where he concluded that the heat resistance exhibited by them was an integral feature of the tertiary and possibly primary structure of the enzyme itself.

Doi (1960) has classified enzymes in the spore as being of two kinds. He states that catalase, alanine racemase, adenosine deaminase, and pyrophosphatase are found to be active without heat activation of the spore. These are located in the outer coats of the spore. On the other hand, enzymes involved in the metabolism of glucose such as alanine dehydrogenase, ribokinase, diaphorase, and several others show much greater activity in spore extracts after an initial heat activation process. These enzymes may be located in the spore coat.

Although enzymes are usually inactive in dormant spores, Church and Halvorson (1957) and Murrell (1955) showed that activated spores of *Bacillus cereus* oxidize glucose in the absence of detectable germination. It has been found possible to activate

a number of enzymes in dormant spores, and this may reflect a general unmasking of cytoplasmic proteins (Sussman and Halvorson, 1966).

Studies of the heat activation of spores (Busta and Ordal, 1964) indicate that rupture of the disulphide bonds in spore enzymes may be an early part of the activation process, and one which is probably dependant on thiol groups (Gould, Hitchins and King, 1966). The enzymes found in Bacillus cereus appear to be bound on or within the central core of the spore, but their release is facilitated by increasing the pH level of the medium, or by raising the solute concentrate (Gould, Hitchins and King, 1966).

O'Connor and Halvorson (1960) noted that there are probably several alternate ways to initiate germination. For example, L-alanine can be replaced by glucose for the germination of many species of spores. Also, the initial stages of germination involve a degradation of the exosporium liberating among other components, L-alanine. Alanine liberated thus could act as an endogenous germinating agent, this germination being inhibited by D-alanine.

The spore enzyme found to be instrumental in the germination of Bacillus cereus may be similar to the spore "S-enzyme" described by Strange and Dark (1957). This enzyme released soluble mucopeptide from the cell walls. Gould and Hitchins (1965) continued

the investigations of Strange and Dark (1957) and found a soluble lytic enzyme which attacked previously treated light phase spores of Bacillus cereus. They concluded that the substrate of the enzyme is probably the structural material of the spore cortex, which is predominantly mucopeptide. The exact mechanism of lytic enzyme release and its action is still unknown. However, if the cortex of the dormant spore is contractile as suggested by Lewis, Snell and Burr (1960), then the action of the enzyme would dramatically terminate the contraction. Or, if the cortex is the site of a permeability barrier (Rode, Lewis and Foster, 1962), this would just as dramatically cease to be effective if acted on by the lytic enzyme.

Sadoff, Bach and Kools (1965) indicate that enzymes within the spore may have some effect on heat resistance. Glucose dehydrogenase appears to be stable within Bacillus cereus spores, but a million-fold change in its heat resistance occurs when the spores germinate.

METABOLIC CHANGES IN ACTIVATION

Activation is a process which causes bacterial spores to germinate in a suitable environment. The term 'activation' in reference to bacterial spores has several connotations. Today, because of new light on the activation process, Murrell (1967) has indicated that the term 'activation' is currently used to

describe two phenomena:

(i) Originally the term 'activation' (e.g. heat activation, Curran and Evans, 1945) was used to describe a treatment which led to an increase in subsequent viable count of a spore suspension.

(ii) Currently the term is used to describe any treatment which leads to an increase in the subsequent rate of germination of a spore suspension whether or not viable count is increased.

In the presentation of the discussion of the results of this thesis, the term 'activation' will refer to the original meaning, that is, treatments which subsequently lead to an increase in viable spore colony count.

In all experiments it was not possible to distinguish between death of spores and induced dormancy. For convenience however a reduction in numbers of viable spores is referred to as lethal effect.

As has already been stated, activation is reversible in most cases, and is based on the change in the macromolecular structure of the spore. Moreover, Keynan and Halvorson (1965) state that this change is not dependent upon metabolic reactions within the spore. The heat-activated spore does not lose its characteristic spore properties, but it changes its qualitative and quantitative requirements for the induction of germination.

Keynan, Issahary-Brand and Evenchik (1965) suggest that activation is basically a change in or a denaturation of the tertiary structure of the protein responsible for the dormancy within the spore, since activation inducers such as heat,

low pH, mercaptoethanol, and thioglycolic acid are found to affect the tertiary structure of proteins. Pollard (1964) noted that the denaturation of protein is usually associated with an unfolding of the secondary and tertiary structure of the protein, and the formation of a new, or a mixture of several new forms, usually less accurately structured than the original.

It is further suggested that the maintenance of the dormant state is caused by a spore coat richly supplied with cystine which is formed in a specific configuration by S-S linkages. Reduction of these linkages would give a change in the tertiary structure resulting in activation, whereas re-oxidation of this bond would bring a return to the dormant state. This theory is borne out by the findings regarding the effects of pH on activation, reported in a later section of this paper. Vinter (1960) elaborates on this theory by stating that the reduction of the cystine-S-S bonds would cause a modification of the structure of the spore-coat protein, thus activating the spores either by increasing the permeability of the spore coat to germinating agents, or by exposure of the enzymes necessary for the germination or by a combination of these effects.

Dormant spores have little or no mRNA, and a defective protein synthesizing system (Kobayashi, Steinberg, Higa, Halvorson and Levinthal, 1965). However, during the conversion of the spore to a vegetative cell, a highly ordered sequence of biosynthetic

events takes place. RNA and protein are synthesized within a few minutes, but it may be a period of one or two hours before DNA synthesis begins (Donnellan, Nags and Levinson, 1965).

Vinter (1965) states that as a spore changes to a vegetative cell, a part of the pre-existing, relatively stable, DAP-containing structure remains in the cells for the whole period of outgrowth, being slowly degraded during this stage. But, the breakdown products of mucopeptides are probably reutilized for synthesis of the new cell wall. Part of the pre-existing envelope structure of the spore can obviously protect the cell up to the stage of elongation, even when the synthesis of the new cell wall is inhibited by antibiotics. As the new cell wall is synthesized, a high penetration of C^{14-} uracil into the cell can be demonstrated, followed by an increase in the incorporation of this compound into ribonucleic acid.

ACTIVATION OF BACTERIAL SPORES

EFFECTS OF HEATING

Heat activation is a process by which a spore population attains a high percentage germination at suboptimal temperatures. Suboptimal temperatures are those which, in the absence of heating, would permit germination of only a small proportion of the total

viable spores. Keynan, Issahary-Brand and Evenchik (1965) note that heat activation has been found essential in conditioning spores for "physiological" (as opposed to chemical) germination in which spores are germinated by a nutrient. Incomplete or delayed germination was frequently observed in the early studies of bacterial spores, until Weizmann (1919) observed that a treatment of 90 - 100°C for 1 - 2 minutes stimulated the germination of spores of Clostridium acetobutylicum. Evans and Curran (1943) and Curran and Evans (1947) were the first to demonstrate systematically that spores which did not germinate or whose germination was delayed in the absence of heat, overcame this "dormancy" when heat-activated.

Most hypotheses concerning the nature of heat activation have been based on the assumption either that (a) heat releases a substance necessary for germination (Levinson and Hyatt, 1960; Levinson, 1961), or (b) that heat inactivates an inhibitor of germination (Juhren, Went and Phillips, 1956). Keynan, et al. (1964) suggested that both of these hypotheses are unlikely. Instead, Vinter's (1960, 1962) finding seems more probable that the macromolecule responsible for maintaining the dormant state is a coat protein rich in cystine, established in a specific configuration by S-S linkages.

Heating has long been known as one of the agents responsible for the denaturation of proteins. In aqueous solutions,

however, the denaturing effects of pH and temperature are so closely connected that the denaturation process can rarely be considered as purely thermal (Joly, 1965).

Evans and Curran (1943) observed that preheating of spores of Bacillus megaterium, Bacillus subtilis, Bacillus cereus, and Bacillus coagulans decreased the time required for germination of these species, and also the minimum temperature at which germ-ination of these species, and also the minimum temperature at which germination would take place. Cook and Brown (1965), working with Bacillus stearothermophilus, found that only about 8% or less of the unheated spores germinated. However, heat activation at 100°C (Fig. 4) increased this by about the same proportion and at the same rate (c. sixfold) as it has been shown to increase colony formation (Cook and Brown, 1964). Even so about 50% of the spores still did not germinate and form colonies even after optimum heat activation. More recent studies have shown that, depending on the germination stimulant employed, heat activation can decrease the time of germination (Sussman and Halvorson, 1966). The effect of heat activation on germination, moreover, varies with the organism, and is influenced by a number of factors including the sporulation medium, the components of the activation medium, and its pH and chemical composition (Curran and Evans, 1945).

An important clue to the nature of heat activation is its temperature dependence. The temperature and duration of optimal

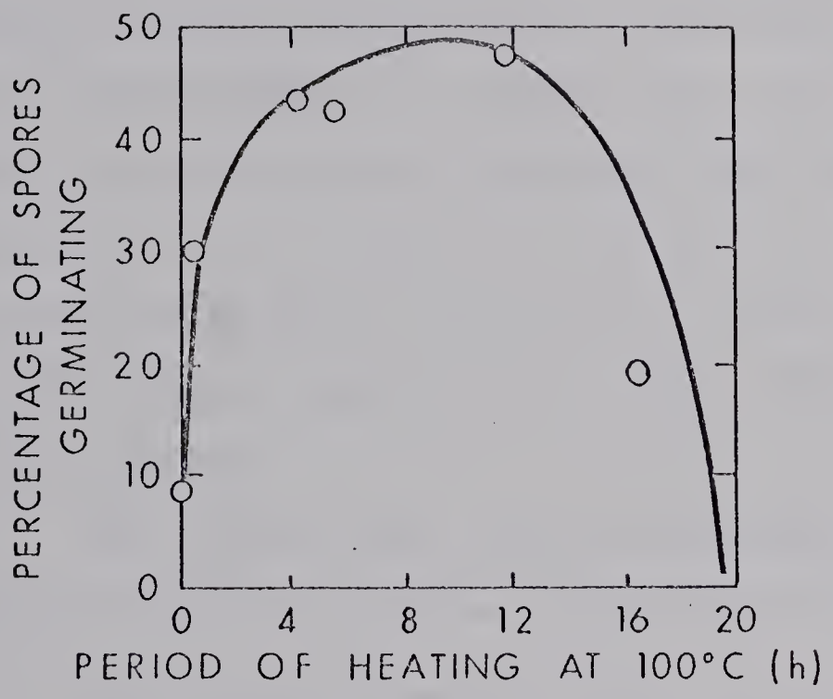

Fig. 4. Effect of heat at 100°C on subsequent germination of spores of Bacillus stearothermophilus (Cook and Brown, 1965).

heating for this effect vary widely among different species and even among different spore preparations of the same strain. Two types of heat dependencies have been reported:

(i) Keynan, Evenchik, Halvorson and Hastings (1964), working with Bacillus cereus strain T, found that for a given time of treatment, there is a critical temperature required, below which activation does not take place. With longer incubation periods, the critical temperature is lowered, but in both cases the temperature range from inactivation to complete activation was only a few degrees, suggesting that the apparent energy of activation is very high.

(ii) Ordal (1964), working with Bacillus subtilis 5230 reported a continuous activation over the temperature range of 5°C to 94°C. Busta and Ordal (1964) felt that the kinetic data indicated little difference between high temperatures heat activation and what has been termed "aging" at low temperatures.

Heat activation induces three measurable changes in the spore suspension:

(i) it increases the germination rate (Church and Halvorson, 1957)

(ii) it activates enzymes which are dormant in the resting spore (Church and Halvorson, 1957)

(iii) it changes the requirement for the induction of germination (Powell and Hunter, 1955).

Brachfeld (1955), after working with Bacillus stearothermophilus, concluded that heat activation was a property primarily

suited to thermotolerant and thermophilic species of bacteria, which possess unusually high thermal resistances. These spores survived heat treatment over 100°C, and this treatment brought about a higher than normal percentage of germination, which greatly increases their potential to cause spoilage in foodstuffs.

Two methods have been commonly used to measure the degree of activation (Keynan, Issahary-Brand and Evenchik, 1965):

(i) Measurement of the rate of germination in a suitable medium. This method is more sensitive for showing differences in the degree of activation of spore suspensions.

(ii) Measurement of the percentage of spores which form colonies when plated on a suitable medium. This method is especially useful for measuring activation in highly dormant spores. The high temperatures needed for activation may kill some spores, so viability as well is checked in this system.

EFFECTS OF pH

Keynan, Issahary-Brand and Evenchik (1965) found that the pH of the medium may determine the rate of heat activation, or whether it will take place at all.

Working with Clostridium bifermentans, Gibbs (1967) noted that activation occurred over a wide range of pH values, but the rate of activation was greater at the extremes of the

range. This latter fact is to be expected if activation is caused by a reversible denaturation-like process of the spore-coat protein as suggested by Keynan et al. (1964). Joly (1965) regarded the pH as particularly determinative in the denaturation of proteins. A great number of proteins are denatured at room temperature by varying the concentration of the hydrogen ions in the absence of other denaturing agents. Joly (1965) suggested a classification of proteins according to their reaction to extremes in pH: some retain structural integrity, some expand, and others dissociate. Some proteins are rapidly denatured at pH values not far removed from the isoionic point; others appear to react reversibly with acid and base over a large pH range.

Keynan et al.(1965) found that optimal heat activation of Bacillus cereus strain T seemed to lie between pH 2.0 - 3.0, although some activation occurred over the range of pH 2.0 - 8.5. They stated that activation is due to the denaturation of protein, and they reinforced this theory with the finding that when pH is between 2.0 and 2.5, carboxyl groups are responsible for changes in the tertiary structure of the protein.

Keynan et al. (1964) took note of the marked activation which took place in Bacillus cereus strain T at a low pH, and suggested that it is not related simply to a breaking of the hydrogen bonds, since high concentration of urea (6M) had no effect on either pH activation or temperature activation.

Keynan, Issahary-Brand and Evenchik (1965) found that a pH above 8.5 inhibited heat activation, but did not damage the spores irreversibly. However, spores heated at a pH below 2.0 for 30 min to 1 hr are permanently changed. Although the spores remained viable, they would not germinate in the usual L-alanine adenosine medium; perhaps this was caused by the irreversible denaturation of an enzyme needed to trigger the spores to react to this medium (Keynan, Issahary-Brand Evenchik, 1965; Gould, Hitchins and King, 1966). When spores heat-activated at a neutral pH were exposed for 30 min to pH 1.0, they lost the capacity to germinate in L-alanine adenosine, but those activated at a low pH for a short time (7 min) did not. Keynan *et al.* (1965) suggested that the irreversible activation which takes place when spores are exposed to pH 1.0 for a short time is caused by either (a) change of the molecular structure of the protein to such an extent that reorganization to the original structure cannot occur, or (b) blocking of the groups necessary for the maintenance of the original structure. Either type of reaction might well take place at the -SH groups, since their oxidation to sulfonic acid would prevent reformation of sulfide bonds, and since exchange reactions between -SH groups are known to occur at a higher rate at a low pH.

Cook and Brown (1965) also noted that the pH of the recovery medium has a profound effect on both heated and unheated

spores. For unheated spores, highest colony counts were obtained in a low pH (5.9) medium, whereas with heated spores, highest colony counts were found in a high pH medium, but as the exposure time was increased, the recovery pattern was reversed. Recovery after severe heat treatment was greatest in a pH 7.3 medium. Presumably as exposure time was increased, the effect of high temperature and high pH cancels the additive force which originally produced high recovery counts, and a reversed action takes place which consequently reverses the recovery pattern, i.e. the effect was lethal to the spores.

The colony counts of heat activated spore of Bacillus stearothermophilus dropped significantly upon storage for 9 mos., but the unheated ones did not (Cook and Brown, 1965). This confirms the findings of Curran and Evans (1947) and Keynan, Issahary-Brand and Evenchik (1965) already mentioned.

Keynan et al. (1964) found that below pH 4.5, the pH dependence of spontaneous activation parallels that of activation induced by reducing agents, whereas between pH 4.5 and 7.2, the activation by reducing agents is increased six-fold.

EFFECTS OF REDUCING AGENTS

Vinter (1960) showed that much of the inert nature of bacterial spores may be attributed to the presence of large numbers of cystine disulphide bonds present in the spore coat of

the protein, and that these are broken during germination. It was possible that reduction of these bonds would cause a modification of the structure of the spore-coat protein, thus activating the spores either by increasing the permeability of the spore coat to germinating agents, or by exposure of the enzymes necessary for germination, or a combination of these effects. Gibbs (1967) treated Clostridium bifermentans spores with mercaptoacetate and found no activation; in fact, a marked inhibition of activation was observed.

Keynan et al.(1964) incubated Bacillus cereus strain T spores both in mercapoethanol and thioglycolic acid, and found that this treatment resulted in the partial activation of the spores. However, heating in the presence of reducing agents served to lower the critical activation temperature only by a degree or two. As well, a minimal time of preincubation with the reducing agent was necessary, since no effect was observed in less than 12 hr.

However, mercaptoacetate is not without some effect on spores of Clostridium bifermentans, since Gould and Hitchins (1965) found that the spores so treated were lysed by hydrogen peroxide or lysozyme. They suggested that the protein-disulphide bonds were involved in the dormancy. They explained the results of Keynan et al. (1964) by assuming that in spores the S-S bonds are nonrandomly distributed, and only some of them are accessible to the added reducing agents.

EFFECTS OF CATIONS

It is known that many ions enhance or inhibit sporulation, but relatively little research has been done regarding their effect on activation. However, Foerster and Foster (1966) have declared ions to be the only universally required agents of physiological germination, organic ions being more widely effective than inorganic.

Lewis, Snell and Alderton (1965) suggest that the Ca^{++} content of spores might be correlated with their degree of dormancy, and they show that activated spores can be reverted to the dormant state by exposing them to Ca^{++} at a high pH. This agrees with the studies of Keynan, Murrell and Halvorson (1961) in which a correlation between dormancy and calcium dipicolinic acid content is shown.

In Bacillus licheniformis spores, calcium apparently inhibits the activation trigger mechanism but not the subsequent germination (Levinson and Sevag, 1953). However, conflicting evidence has appeared. In 1962, Halmann and Keynan suggested that divalent cations might be necessary for the activation of dormant Bacillus popilliae spores, since their activity was enhanced by calcium and other divalent ions, and inhibited by chelators of the same. Working with the same organism, Splittstoesser and Farkas (1966) found maximum activation rates in a calcium solution at pH 7.0. Their further finding that a low pH with calcium inhibited activation has perhaps given a clue to the role of calcium and other

ions in activation. They state that calcium probably affects the later stages of germination, and that its role is greatly affected by the pH of the medium. Rode and Foster's (1966) results with Bacillus megaterium Texas showed similar results to those of B. popilliae. However, Splittstoesser and Farkas (1966) found that potassium had an inhibitory effect on activation, probably because of the competition with calcium for some active site.

Keynan et al. (1965) stated that although calcium seems to be a factor in dormancy, it is not the only factor. This is indicated by the findings of Halmann and Keynan (1962) in which the addition of salts to a spore medium inhibited activation. In this experiment, the spores had been washed only once and suspended in a buffer to germinate. Apparently, salts are adsorbed on spores during heating and can be removed only by thorough washing. After such washing, activation was found to be more rapid due to the introduction of salts to the initial medium.

Ca^{++} ions have been shown to protect proteins from heat, and might have a similar effect on the hypothetical spore protein which keeps the spores dormant (Keynan et al., 1965).

Even in the absence of multivalent metallic ions, the action of various salts is often comparable with a denaturation of protein. Joly (1965) found that the optical rotation of collagen

varies from -350° to -80° by the addition of $CaCl_2$, potassium iodide, and potassium thiocyanate. This denaturation is reversible in the case of $CaCl_2$. The action of these salts has been attributed to the solvation effect. Joly (1965) concluded that salts increase the rate of protein denaturation below pH 4.8, but decrease or inhibit it above this pH, these alterations being proportional to the ionic strength.

MATERIALS AND METHODS

ORGANISMS

The test organisms were Bacillus subtilis strain 8057 736NCIB-8057 (3R9675; ATCC9524; NRRLB-314), NCIB, 1955; and Bacillus cereus strain 127, 578 Bacillus cereus BC127. M.J. Stone, NIRD, 1952. It produces bitty cream in milk. J. Dairy Research, 20, 29, 1953, which were obtained from the National Collection of Dairy Organisms, National Institute of Research in Dairying, University of Reading. These strains were used because they had excellent spore producing capabilities.

MEDIA

Five media were examined to determine which would produce maximum sporulation with these organisms in the minimum time:

(i) A solid medium (Williams, Franklin, Chapman and Clegg, 1957a; Whitehouse and Clegg, 1963) which contained manganese to stimulate spore production, and was made up as follows:

Bacto tryptone	3 g
Bacto peptone	6 g
Yeast extract	3 g
Beef extract	1.5 g
Agar	25 g
Solution containing 0.001% w/v Mn as $MnSO_4$	1 ml
Distilled water to	1 liter

(ii) Tryptone-glucose-extract agar (Difco) reinforced by adding 2 g $CaCl_2$.

(iii) Minimal synthetic medium. The glucose-glutamic acid-glycine synthetic medium was first used by Lundgren and Beskid (1960) and was composed as follows:

Glucose	5	g	KH_2PO_4	0.5	g
Glutamic acid	10	g	$MgSO_4.7H_2O$	0.2	g
Glycine	0.1	g	$FeSO_4.7H_2O$	0.01	g
$ZnSO_4.7H_2O$	0.013	g	$MnSO_4.4H_2O$	0.01	g
K_2HPO_4	0.5	g	$CaCl_2$	.1	g/l

(iv) Nutrient broth (Difco).

In the use of each of the above media, microscopic examination showed sporulation to be satisfactory, but each presented a major disadvantage in that the required 7 - 14 days for maximum sporulation at 37°C.

(v) Nutrient agar was found to be most satisfactory. This basic nutrient agar (Curran and Evans, 1945) was fortified with 0.5 g $CaCl_2$ and .001% w/v Mn^{++} as $MnSO_4$, and gave virtually complete sporulation in 36 - 48 hr incubation at 37°C. This medium was used continuously every two weeks to produce stock spore suspensions.

SPORE PRODUCTION - BACILLUS SUBTILIS 8057

The nutrient agar medium used for spore production of Bacillus subtilis 8057 was dispensed in 100 ml quantities in Roux bottles and sterilized for 20 min at 121°C. The inoculum, from a stock culture sample, had previously been cultivated on B.B.L.

trypticase soy broth at 37°C, and transferred every 24 hr, for a three day period. When microscopic examination showed the vegetative cells to be actively multiplying, the suspension was aseptically pipetted in 1 ml quantities into the Roux bottles. The bacterial suspension was evenly distributed on the surface of the medium by tilting the bottle. The Roux bottles were incubated at 37°C for 36 - 48 hr. Throughout this time, slides were made periodically and examined to determine the degree of sporulation.

When sporulation was virtually complete, the Roux bottles were stored in the refrigerator at 4°C for a period of 18 hr. This procedure was found to be effective in producing spores which were comparatively free of sporangia. This procedure was also effective in bringing about lysis of most of the vegetative cells present on the surface of the medium. This considerably simplified the purification procedure.

Following refrigeration the growth was harvested from the agar surface by sterile distilled water and sterile glass rod scrapers. The suspensions so formed were decanted into sterile centrifuge tubes and centrifuged at 10,000 rpm for 1 min, and the supernatant fluid decanted. This procedure involved a considerable loss of spores which either remained in suspension in the supernatant liquid, or adhered to the sides of the centrifuge tubes. This was remedied by using TWEEN 80 (Vinter, communicated to

Splittstoesser and Farkas, 1966) which worked satisfactorily and gave much better spore recovery during the washing procedure.

PURIFICATION OF THE SPORE SUSPENSION BY LYSOZYME METHOD

Previous workers, Williams, Clegg and Wolf (1957) killed vegetative cells in spore suspensions by immersing the whole suspension in a water bath at 80°C for 20 min. This was tried but it was felt that this introduced preheating of the spores which was undesirable in work on heat activation.

Finley and Fields (1962) used the lysozyme method of purifying spore suspensions. The spores were resuspended in 50 ml of distilled water and transferred to sterile flasks containing a magnetic stirring rod. A 0.5 mg/ml concentration of lysozyme 3X* was added to each suspension and the mixtures were incubated at 52°C for 2 hr during which time the suspension was agitated with a magnetic stirrer. Microscopic examination showed that spores were completely free from the sporangia and that vegetative cell debris was completely lysed into fine particles.

The spore suspension was washed by centrifuging five times at 3000 rpm for 20 min to free it of lysozyme and particulate impurities. To break up spore clumps, this washed suspension was shaken for 2 hr with glass beads in a cooling room at 6 - 14°C.

The spore material was separated by centrifugation for

*Nutritional Biochemicals Corporation

20 min at 3000 rpm (1465 g) for the initial separation and, for each subsequent separation, at a speed of 500 rpm greater than the previous time up to a maximum of 8000 rpm (10400 g), as described by Busta and Ordal (1964). The clean spores were suspended in 100 ml deionized, sterile, distilled water, phosphate buffered to pH 7.0, and stored at 4°C in screw cap bottles.

This stock suspension showed a concentration of spores of the order of $1x10^{8}$ /ml. This was obtained from a total count with the use of a Petroff-Hauser counting chamber. The total viable spore count from this suspension was obtained by serial dilution in phosphate-buffered water blanks and plating, and this was found to be in the range of 6 - $8x10^{7}$/ml.

RECOVERY MEDIA

The recovery medium was that on which the treated spores were inoculated to check for viable spores. The two media which gave most satisfactory results were:

(i) Nutrient Agar

(ii) Plate Count Agar (PCA).

The Nutrient Agar gave distinct colonies which did not show great spreading within 24 hr. but the recovery count was not as high as that of the Plate Count Agar. The Plate Count Agar, while producing more colonies, allowed considerable spreading after

24 hr. The Plate Count Agar was chosen because of the higher colony counts; difficulties with spreading were minimized by counting the plates at 18 - 24 hr.

EXPERIMENTAL MATERIALS

Oil Bath

Oil baths were used containing polyalkylene-glycol (Canadian Carbide), with a flash point of 260°C, in preference to water baths because of the low boiling point of water (usually in the range of 94 - 96°C) at the elevation of the city of Edmonton, ca. 2000 ft.

The oil baths were tightly fitted with pressed fiberboard lids and holes, which could accommodate size 3 rubber stoppers, were drilled in the fiberboard. The rubber stoppers were fitted with screws to which a six inch length of soft wire was attached.

The sealed heating ampules containing the spore suspension were attached to the free end of the wire, so that they were completely submerged during the experiment. Even though the oil bath contained a fixed dial thermometer within its elements, its reading did not always correspond with the temperature reading of the polyalkylene-glycol medium. Therefore, a tested thermometer was permanently fixed through the fiberboard, and its reading was always used as the accurate temperature

of the heating medium.

Ampules

Five ml thin glass ampules were used throughout this investigation. One ml quantities of the spore suspension were introduced into these ampules by means of sterile disposable syringes. The ampules were sealed by heat for complete immersion in the heating medium.

Syringes

Sterile disposable plastic syringes were used, with a capacity of 2.5 ml, which were fitted with 22G needles, and cylinders graduated in centimeters and inches. These syringes were purchased in sterile packs; tests for sterility were consistently satisfactory.

Dilution Blanks

Two liter quantities of dilution liquid were prepared. This was buffered with 1.25 ml/l of phosphate buffer* and the pH was adjusted to 7.4 before autoclaving. This solution was then dispensed in 9.5 ml quantities in test tubes, 40 to a basket. The test tubes were then autoclaved at 121°C for 20 min after which the pH dropped to 7.0 - 7.2, and the volume was reduced to the required 9.0 ml. After autoclaving, the dilution blanks were cooled and stored at 5°C.

* Prepared from 34 g KH_2PO_4 dissolved in 500 ml distilled H_2O Lab. Manual, 1964.

Recovery Medium

As previously mentioned, the recovery medium used was Plate Count Agar, which was prepared in 1 liter quantities, pH adjusted to 7.4, dispensed in 10 ml quantities into test tubes and sterilized at 121°C for 20 min. After autoclaving, the pH was 7.0 - 7.1. The medium was cooled and stored. Fresh medium was made up every 2 days.

Petri Dishes

Plastic Petri dishes were used for plating suspensions in preference to glass dishes because of convenience. The sterility of the plastic Petri dishes was tested and found to be satisfactory. A comparison between glass and plastic Petri dishes was made, and because the glass dishes frequently had concave bases, which encouraged concentration of colonies in the central part of the dish, plastic dishes were preferred.

Pipets

Delivery, white, Belco, 1 ml pipets were used throughout for dilution, and for inoculating the recovery plates.

SPORE PRODUCTION-BACILLUS CEREUS 127

All methods used for Bacillus cereus 127 were the same as those used for the production of spores of Bacillus subtilis 8057, except that on two occasions during the purification process

for B. cereus 127, it was found that no spores were left in the pellet, i.e. all of the spores were being discarded with the supernatant liquid. Microscopic examination of the supernatant showed a good collection of clean spores in this suspension.

Toda and Aiba (1966) found that the specific gravity of bacterial spores ranged from 1.05 to 1.235, depending on the species. In this experiment, the spores did not form pellets during centrifugation, but remained suspended in the supernatant. Toda and Aiba (1966) found the specific gravity of B. cereus to be 1.2. However, they have not mentioned the strain, and it is possible that the B. cereus 127 has a density below or equal to that of water, which accounts for the fact that its spores remained in suspension.

Thus, in subsequent preparation of spores of B. cereus 127 for experimental work, instead of discarding the supernatant, it was saved in screw-cap bottles after each successive washing by centrifugation. This gave an abundance of evenly distributed spores in suspension, free from cast-off vegetative cells and other debris, which, in reality, formed the pellet. This procedure made purification and washing of spores very easy.

COUNTING METHOD

Two methods were compared, the Spot Plate Technique for viable cell counts (Gaudy, Abu-Niaaj, and Gaudy, 1962), and the

Pour Plate Technique.

Gaudy (1955) found the Spot Plate Technique successful in studying aerobic bacterial species. The method involves application of a known volume of a bacterial suspension onto the surface of solidified agar. The bacterial count was based upon a total sample volume of 0.08 ml applied in four 0.02 ml spots. Four spots can easily be placed on the agar surface. This technique was abandoned after repeated trials, because of the strong tendency of colonies of aerobic spore-formers to coalesce.

Further as the pour plate technique was statistically comparable to the spot plate method (Gaudy, 1955), the pour plate technique was used for the entire experimental study, with three replicate plates for each count.

CONTAMINANTS

At one period of several weeks in the experimental investigation, the plate counts were ruined by contaminants. By a process of elimination, the contamination was pinpointed as coming from the PCA which, at that time was being prepared in 100 ml quantities, contained in screw cap bottles. It was found that the bulk quantity did not receive complete sterilization from the laboratory autoclave. Consequently, the melted plating medium was dispensed in 10 ml quantities in screw cap test tubes and autoclaved, and this was found to be completely satisfactory.

RELIABILITY OF RESULTS

To ascertain the reliability of the plating technique, and also to note the significant differences among the average viable spore counts, the Standard Error of the Mean was calculated in all cases that were pertinent for intelligent interpretation of the results. The formulae used were as follows:

$$\text{Standard Deviation} - S = \sqrt{\frac{\Sigma X^2 - \frac{(\Sigma X)^2}{n}}{n - 1}}$$

$$\text{Standard Error of the Mean} - (S\bar{x}) = \frac{S}{\sqrt{n}}.$$

Each experiment shown in the Figs. or Tables was repeated at least once. Wherever a characteristic trend was commented on in a curve this had been verified from a previous experiment and the examples given are typical examples.

The difference between the volume spore suspension in each ampule was 4.8%.

EXPERIMENTAL PROCEDURE AND RESULTS

EFFECT OF HEAT ON THE ACTIVATION OF SPORES

Procedure

One ml quantities of the stock suspension were dispensed in ampules with sterile, disposable syringes. The ampules were completely immersed in the heating medium, as previously described, and received heat treatments ranging from 65 - 115°C at 5°C intervals. Each temperature was used over a time range of 5 - 20 min at 5 min intervals, and 20 - 100 min at 20 min intervals. Following this, the ampules were removed, cooled to room temperature (21 - 38°C), opened aseptically, and the suspension transferred by a syringe to phosphate-buffer blanks. Serial decimal dilutions were made up to 10^{-6}. Petri dishes were inoculated in triplicate and incubated at 37°C. Plates were counted after 18 - 24 hr incubation.

Results

Table 3 and Fig. 5 show the effect of heat on the activation of spores of Bacillus subtilis 8057. The vertical lines above and below a plot indicate the standard error of the mean ($S\bar{x}$). Spores of Bacillus subtilis 8057 were given heat treatments for 10 min (pH 7.0) within the temperature range of 65 - 115°C with an interval of 5°C. In the case of temperatures below 85°C, the viable spore colony count was below that of the

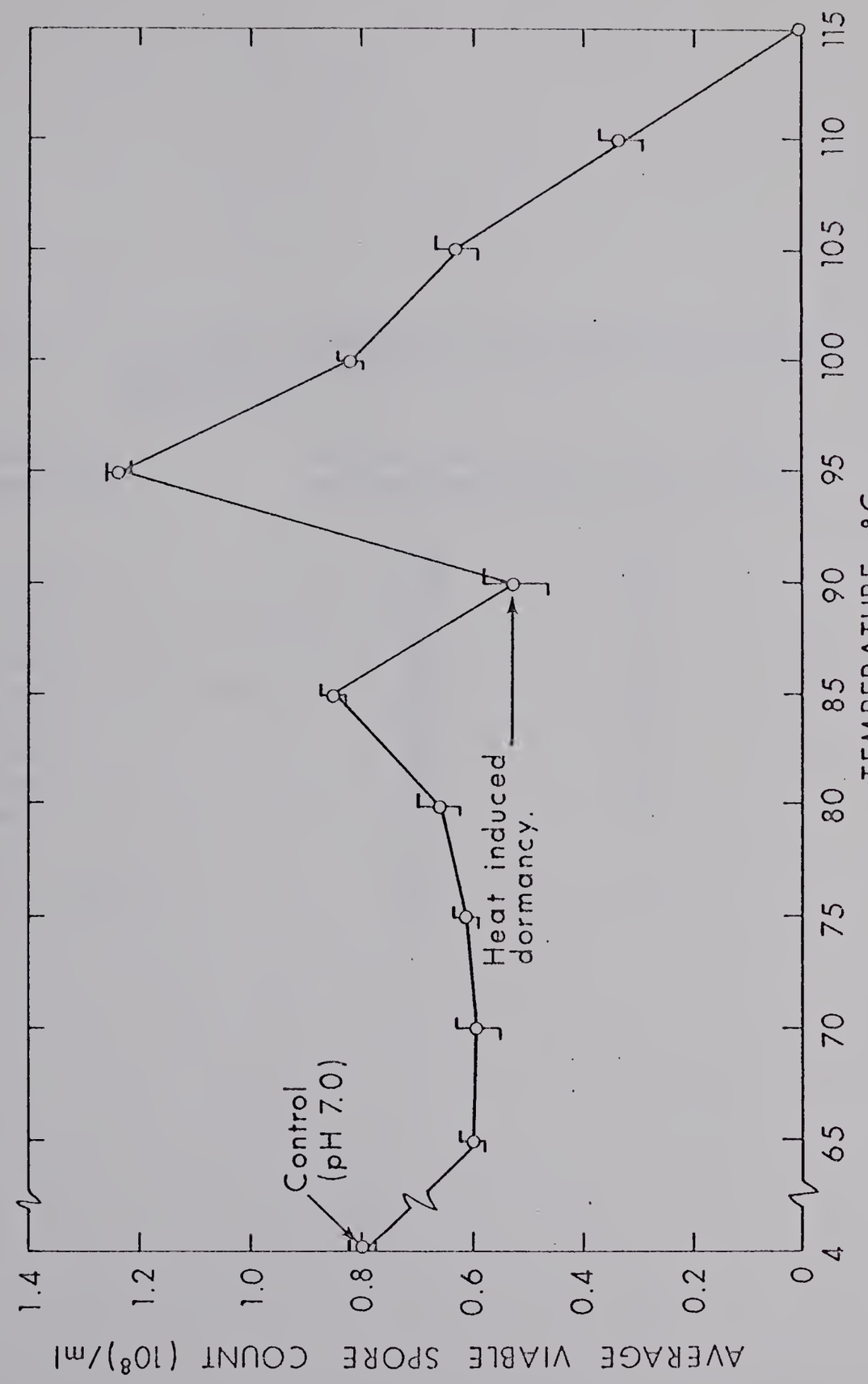

Fig. 5. The effect of different heat treatments for 10 min at pH 7.0 on germination of spores of Bacillus subtilis 8057.

Table 3. The effect of different heat treatments for 10 min at pH 7.0 on germination of spores of Bacillus subtilis 8057.

Heating temp (°C)	Average viable spore count (10^6)/ml and Standard Error of the Mean ($S\bar{x}$)	
65	60	±0.6
70	59	2.5
75	61	1.1
80	66	2.3
85	85	1.2
90	52	3.4
95	124	1.0
100	82	1.1
105	63	1.8
110	33	2.0
115	0	-
Control (4°)	80	1.12

control with no heating, whereas 85°C gave viable spore colony counts comparable to the control ($S\bar{x} \pm 1.02$). Heat treatment at 95°C gave activation at which point the viable spore count was significantly greater than the control. At temperatures beyond 95°C, there is an abrupt decrease in colony count. The temperature of 105°C is the critical temperature at which the lethal effect is evident. Within the temperature range of 85 - 95°C, there is a significant drop in colony count at 90°C. This phenomenon has been observed and reported by several workers studying the effect of heat and radiation energy on bacterial spores, and several names have been given to describe it, e.g. "heat induced dormancy" (Finley, 1964), and "paradoxical inversion", in the case of radiation energy (Grecz, 1965).

A temperature of 95°C for 10 min at a pH of 7.0 gave a maximum activation of B. subtilis 8057 spores suspended in a phosphate buffer. Thus, it was of further interest to see whether an increase in the time of treatment at different temperatures would have any effect on activation. Fig. 6 and Table 4 show the effect of duration of heat treatment (5 - 100 min) at various temperatures (80 - 110°C), maintaining all conditions similar to the previous experiment, on the recovery of B. subtilis 8057. The temperature of 95°C was definitely superior for activation to the rest of the temperatures in the range mentioned above;

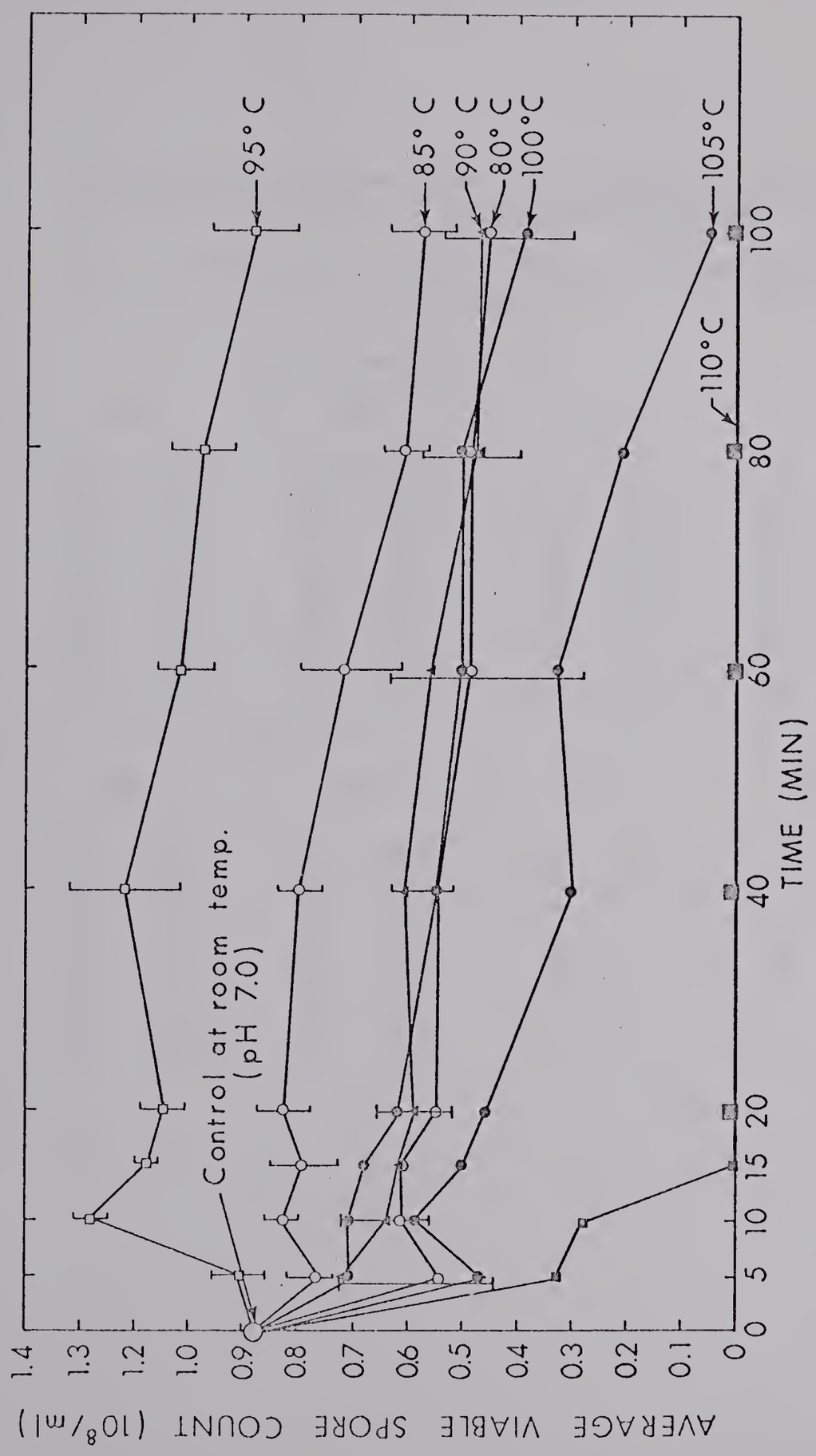

Fig. 6. The effect of different time-temperature treatments on germination of spores of Bacillus subtilis 8057.

Table 4. The effect of different time-temperature treatments on germination of spores of Bacillus subtilis 8057.

Time (min)	Average viable spore count (10^6)/ml and Standard Error of the Mean ($S\bar{x}$)							
	Temperature °C							
	80		85		90		95	
5	54	±3.7	77	±2.4	72	±4.7	91	±2.6
10	61	2.1	83	1.5	64	3.0	128	1.5
15	61	1.4	79	2.1	62	1.8	118	0.9
20	55	3.0	83	2.5	59	1.4	115	2.0
40	55	1.1	80	1.8	61	1.1	122	4.9
60	49	2.0	72	5.0	56	1.8	102	2.3
80	49	3.0	61	2.3	48	4.7	98	2.7
100	46	3.5	58	3.5	47	3.4	89	4.3
	100		105		110		115	
5	71	±4.2	47	±5.2	33	±1.8	0	-
10	71	1.5	59	2.1	28	0.9	0	-
15	68	3.6	50	2.3	0	-	0	-
20	62	1.5	46	7.5	0	-	0	-
40	55	4.6	30	5.5	0	-	0	-
60	50	4.5	33	2.1	0	-	0	-
80	50	2.1	21	3.5	0	-	0	-
100	39	1.8	5	0.8	0	-	0	-
Control (4°)	88	3.6						

that is to say that at every duration of heat treatment, 95°C gave a maximum viable spore colony count significantly greater than that of the control. Temperatures of 80, 90, and 100°C did not yield much difference in viable spore count over all exposure times. The temperature of 85°C gave significantly greater results than temperatures of 80, 90, and 100°C, but the average viable colony counts were always less than that of the control. Again, Fig. 6 shows, as in Fig. 5, that 105°C is the critical temperature which results in a noticeable abrupt decrease in colony count beyond 10 min of treatment.

Spores of B. cereus 127 were given heat treatments for 10 min (pH 7.0) within the temperature range of 60 - 115°C with increments of 5°C. Fig. 7 and Table 5 summarize the results. In the case of this organism, the viable spore colony count with all heat treatments was always below that of the control, with the exception of 65°C where there was no difference; in other words this organism did not respond to heat activation. In view of this, it was considered that increased duration of heating might produce activation. Therefore, an experiment was done in which spores were heated from 5 - 100 min in intervals of 5 min up to the 20th min, and then at intervals of 20 min. Spores were heated at temperatures ranging from 65 - 100°C at intervals of 5°C. The results are expressed in Fig. 8 and Table 6. At 65°C (between 10 and 80 min), the viable spore colony counts exceeded or equalled that of the

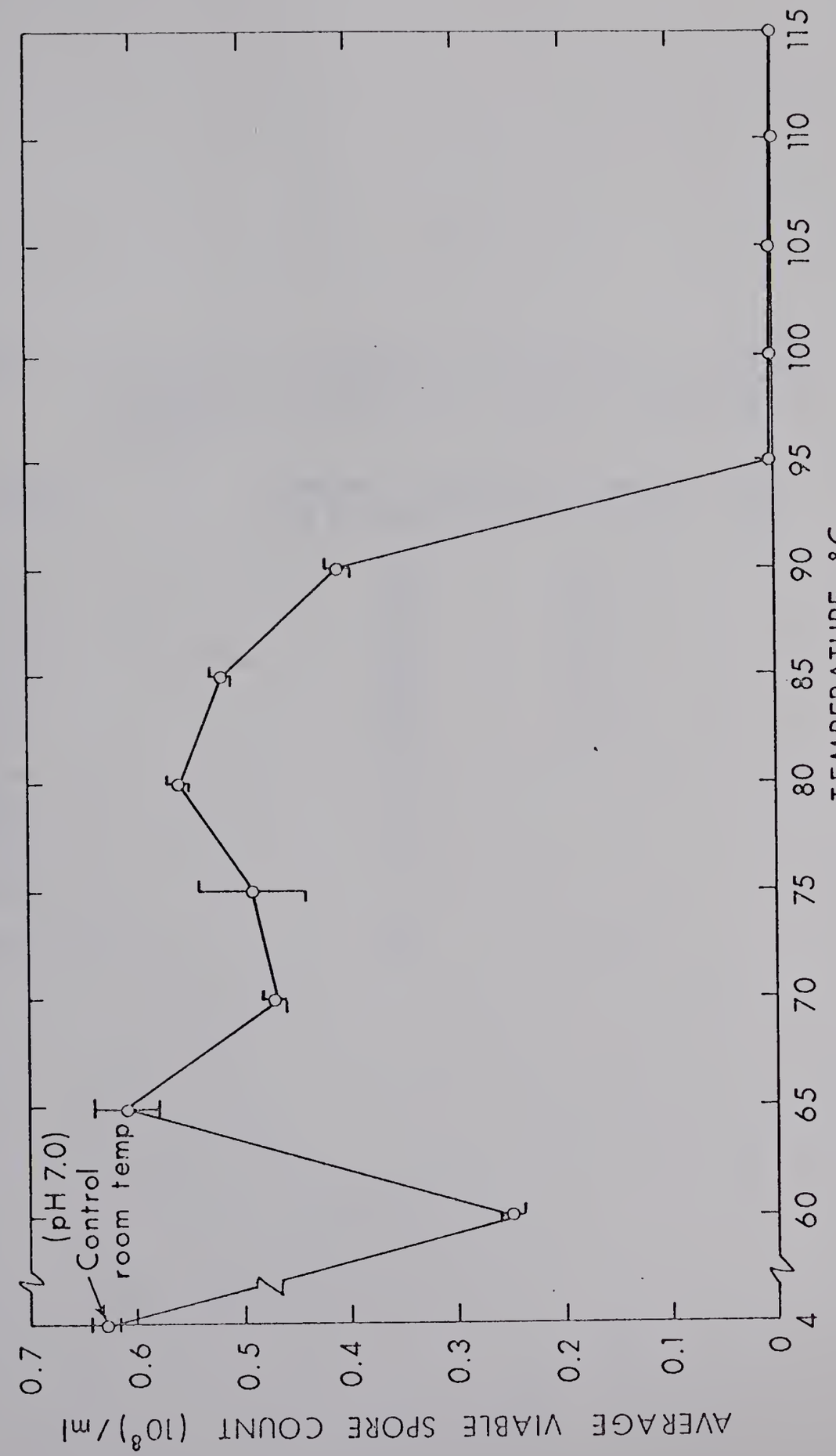

Fig. 7. The effect of different heat treatments for 10 min at pH 7.0 on germination of spores of Bacillus cereus 127.

Table 5. The effect of different heat treatments for 10 min at pH 7.0 on germination of spores of Bacillus cereus 127.

Heating temp (°C)	Average viable spore count (10^6)/ml and Standard Error of the Mean ($S\bar{x}$)	
60	25	±1.5
65	62	2.7
70	47	1.4
75	49	5.4
80	56	0.9
85	52	1.1
90	41	0.7
95	0	-
100	0	-
105	0	-
110	0	-
115	0	-
Control (4°)	63	1.12

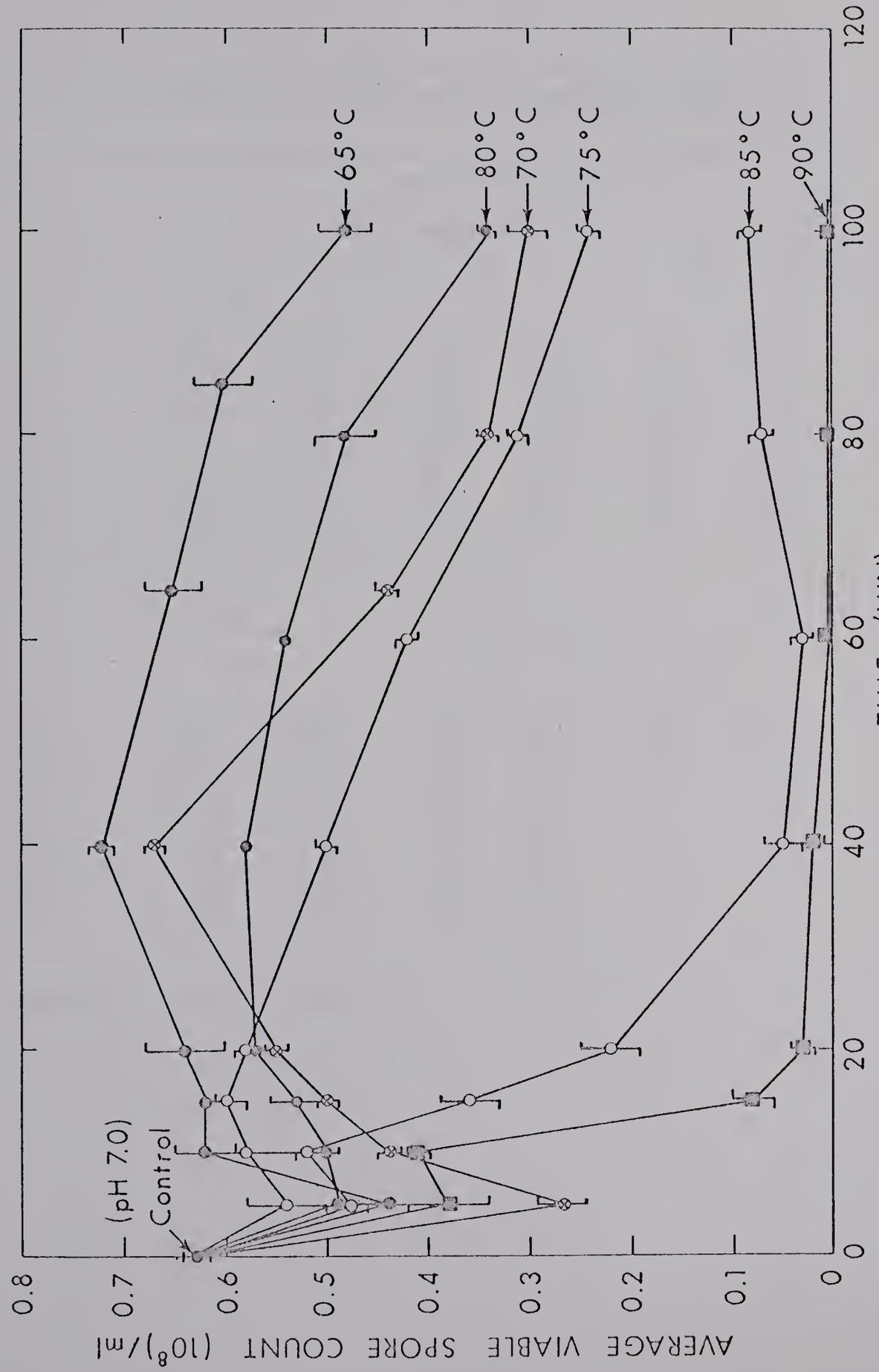

Fig. 8. The effect of varying the time-temperature treatments on germination of the spores of Bacillus cereus 127.

Table 6. The effect of varying the time-temperature treatments on germination of the spores of Bacillus cereus 127.

Time (min)	Average viable spore count (10^6)/ml and Standard Error of the Mean ($S\bar{x}$)					
	Temperature °C					
	65		70		75	
5	44	±2.1	27	±2.6	49	±4.3
10	62	2.7	49	1.4	50	5.4
15	62	1.4	50	0.6	53	2.3
20	64	4.7	55	0.6	57	0.6
40	72	0.8	62	1.1	58	1.2
60	65	3.6	44	0.7	54	0.6
80	60	3.0	34	0.6	48	1.0
100	48	2.9	30	2.0	34	0.9
	80		85		90	
5	54	±0.9	48	±3.5	38	±2.6
10	58	0.9	52	1.1	41	0.7
15	60	2.7	36	3.2	8	1.7
20	58	0.6	22	3.2	3	0.9
40	50	0.3	5	2.1	2	1.0
60	42	0.3	3	0.9	0	-
80	31	3.5	7	1.0	0	-
100	24	1.3	8	1.0	0	-
Control (4°)	63	1.02				

control. However, this was really mild activation, and only at 40 min did significant activation occur. A temperature of 70°C gave significant activation at 40 min; however all other times of treatments gave results inferior to the control. All other temperatures gave viable spore colony counts that were consistently below the control also, indicating a gradual thermal destruction of the spores, as exposure times were increased. Temperatures of 85°C and 90°C show a drastic thermal destruction of the spores, which occurred immediately after 10 min of treatment.

EFFECT OF pH ON THE ACTIVATION OF SPORES

Procedure

Glycine was used to prepare solutions of pH 2.0 to pH 10.0 in intervals of pH 1.0. The exact pH was adjusted with 6N HCl or NaOH. Each adjusted pH solution was autoclaved and stored in screw cap bottles. It was found that autoclaving had no reduction effect on each adjusted pH.

Nine ml quantities of the adjusted pH solution were dispensed in screw cap culture tubes. To each of these, 1 ml of the stock spore suspension was added, and the mixture agitated with a Vortex mixer. The mixture was allowed to stand for a few minutes to equilibrate to room temperature (21 - 38°C). From this mixture 1 ml quantities were dispensed into ampules with sterile syringes. The ampules were sealed and then heated for 10 min at 95°C.

The findings of the above 10 min treatment were used to plan an experiment to investigate the effect of pH and heat at different times of exposure, as follows:

(i) pH and heat - 1 ml of the spore-pH suspension were introduced into ampules, sealed and heated in the oil bath at 65 - 95°C with 5°C intervals for periods of 2, 5, 10, and 15 min.

(ii) pH alone)
(iii) Control alone) Method as given above (i)
(iv) Control and heat)

The control consisted of 1 ml of the stock spore suspension which received no adjustment of pH, and which was plated out under the same conditions as the treated samples. Plates were prepared to 10^{-6} dilution and incubated at 37°C for 24 hr.

Results

The results of this experiment with B. subtilis 8057 are summarized in Figs. 9, 10 and Table 7.

The temperature of 65°C showed no activation since the plate counts of viable spores were significantly below that of the control throughout the pH range. pH 2.0 showed lethal effect on the spores at 70°C, and throughout the temperature range. Temperatures of 70° - 80°C gave distinct activation from pH 6.0 to 10.0. The treatment 75°C with pH values of 6.0, 7.0, 8.0, 9.0, and 10.0 gave activation that was significantly above the control. At 80°C and a pH of 9.0 and 10.0, activation was also significantly

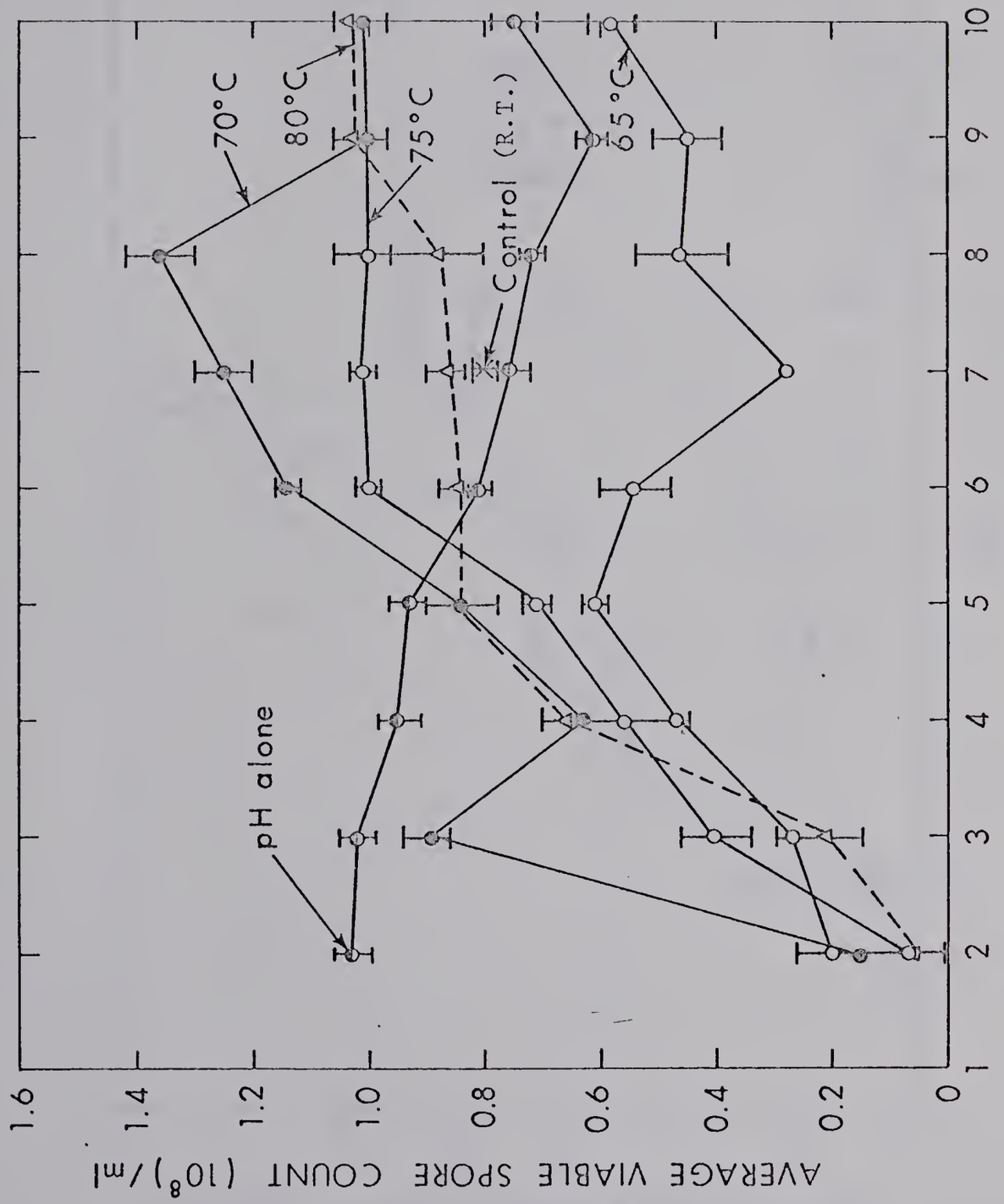

Fig. 9. The effect of pH and heat at varying temperatures for 10 min on germination of spores of Bacillus subtilis 8057.

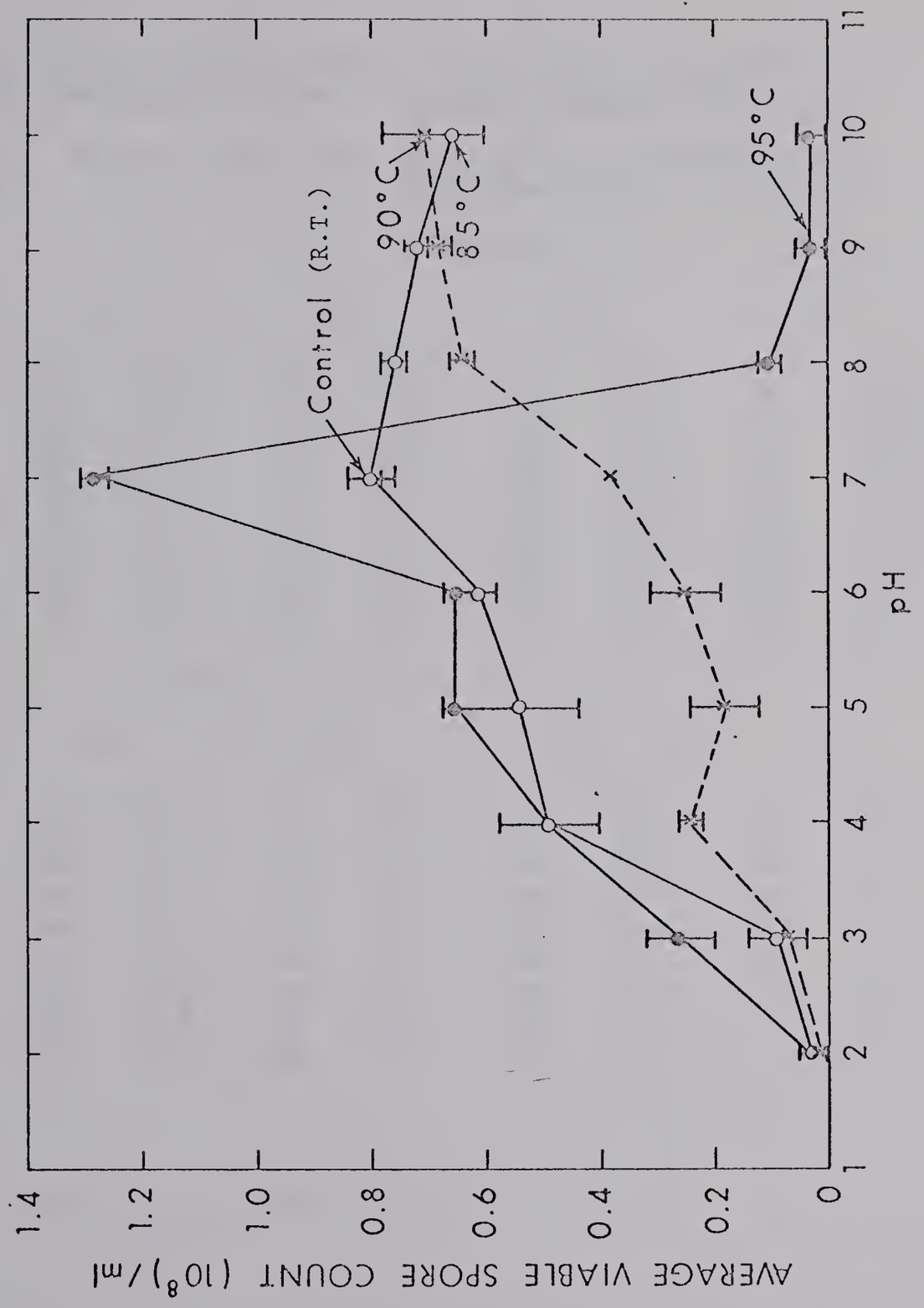

Fig. 10. The effect of pH and heat at varying temperatures for 10 min on germination of spores of Bacillus subtilis 8057.

Table 7. The effect of pH and heat at varying temperatures for 10 min on germination of spores of Bacillus subtilis 8057.

pH of heating menstruum	Average viable spore count (10^6)/ml and Standard Error of the Mean ($S\bar{x}$)							
	Temperature °C							
	R.T.		65		70		75	
2	103	±1.6	20	±2.7	15	±2.0	7	±0.7
3	102	1.4	27	1.2	89	2.2	40	3.2
4	95	2.0	47	0.7	63	2.9	56	4.1
5	93	1.7	61	0.9	84	2.8	71	1.1
6	81	1.5	54	2.6	114	1.0	102	1.1
7	76	2.8	27	0.3	125	3.2	105	1.7
8	71.6	1.2	46	4.1	136	2.8	102	3.0
9	61	1.3	45	2.9	105	0.9	100	2.3
10	75	3.0	58	2.0	108	2.5	115	3.5
	80		85		90		95	
2	6	±2.3	3	±0.9	2	±0.3	3	±0.6
3	21	3.2	9	2.3	7	0.3	26	3.0
4	66	2.0	49	1.7	24	0.9	49	4.5
5	84	2.2	54	5.2	18	3.0	65	1.1
6	84	1.1	61	1.5	25	2.6	65	1.1
7	86	2.0	80	2.2	38	0.3	128	1.1
8	88	2.0	76	1.4	64	1.2	10	1.1
9	118	2.0	72	1.1	68	1.0	3	1.5
10	116	1.5	66	2.7	70	4.1	3	1.5
Control at pH 7.0 and 4°	80	1.6	63	1.02				

above that of the control. But, most dramatic effect was seen with 70°C between pH 5.0 and 10.0, with peak point of activation at pH 8.0.

Temperatures 85°C and 90°C (Fig. 10) gave viable spore counts that were consistently below that of the control throughout the pH range 2.0 - 10.0, indicating the lethal effect of the treatment on the spores. A temperature of 95°C gave maximum activation at pH 7.0. However, all other pH treatments below 7.0 were drastically lethal.

In view of the fact that there were two activation peaks, namely pH 8.0 at 70°C, and pH 7.0 at 95°C, it was considered appropriate to investigate what effect different duration times would have at these temperatures and the appropriate pH. Accordingly, duration times of 2, 5, 10, and 15 min were further investigated. At 70°C, pH 6.0, 7.0, and 8.0 were studied at different times, and Fig. 11 and Table 8 summarize the results. With treatments of pH 7.0 and 8.0 all exposure times resulted in activation with a peak at 10 min whereas with pH 6.0 the only time which showed activation was 10 min.

Fig. 12 and Table 9 show that with the exception of pH 4.0 and 5.0, at treatment times of 2 and 5 min all treatments were progressively lethal as the time was increased.

Fig. 13 and Table 9 indicate that all pH treatments with the exception of pH 7.0 interfered with heat activation at some or all of the

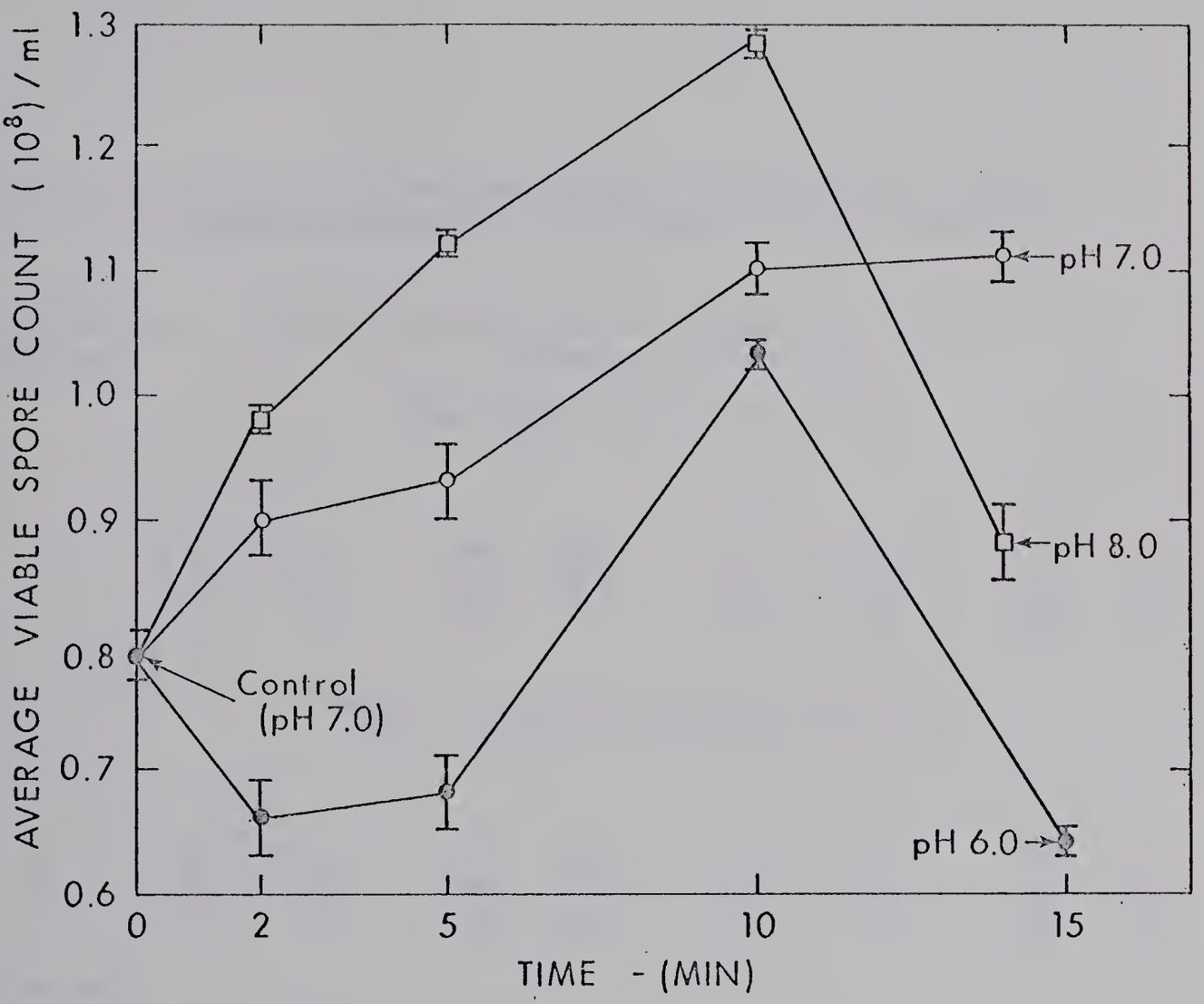

Fig. 11. The effect of pH and heat (70°C) on germination of spores of Bacillus subtilis 8057 at different exposure times.

Table 8. The effect of pH and heat (70°C) on germination of spores of Bacillus subtilis 8057 at different exposure times.

pH of Heating Menstruum	Average viable spore count (10^6)/ml and Standard Error of the Mean ($S\bar{x}$)							
	Time (min) of exposure at 70°C							
	2		5		10		15	
6	66	±3.5	68	±3.0	103	±0.9	64	±0.9
7	90	3.5	93	2.6	110	2.0	111	2.0
8	98	1.2	112	1.4	128	1.2	88	6.8
	Time of exposure at room temperature							
	2		5		10		15	
6	68	±2.9	79	±3.3	78	±4.6	71	±1.7
7	71	1.8	87	1.7	80	0.5	84	0.5
8	91	1.4	86	0.6	79	0.6	81	0.6
Control at pH 7.0 and 4°	79	1.8						

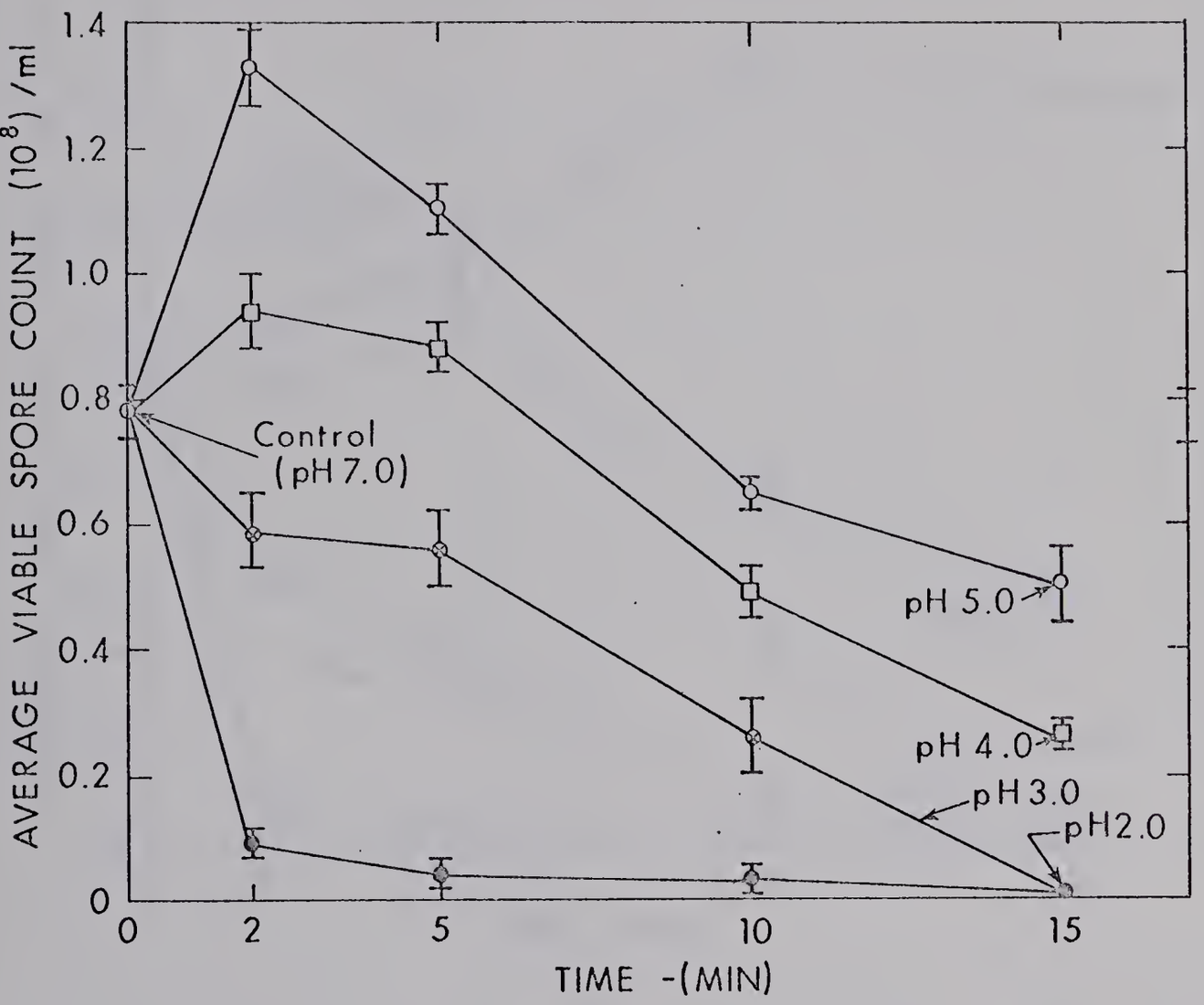

Fig. 12. The effect of pH and heat (95°C) on germination of spores of Bacillus subtilis 8057 at different exposure times.

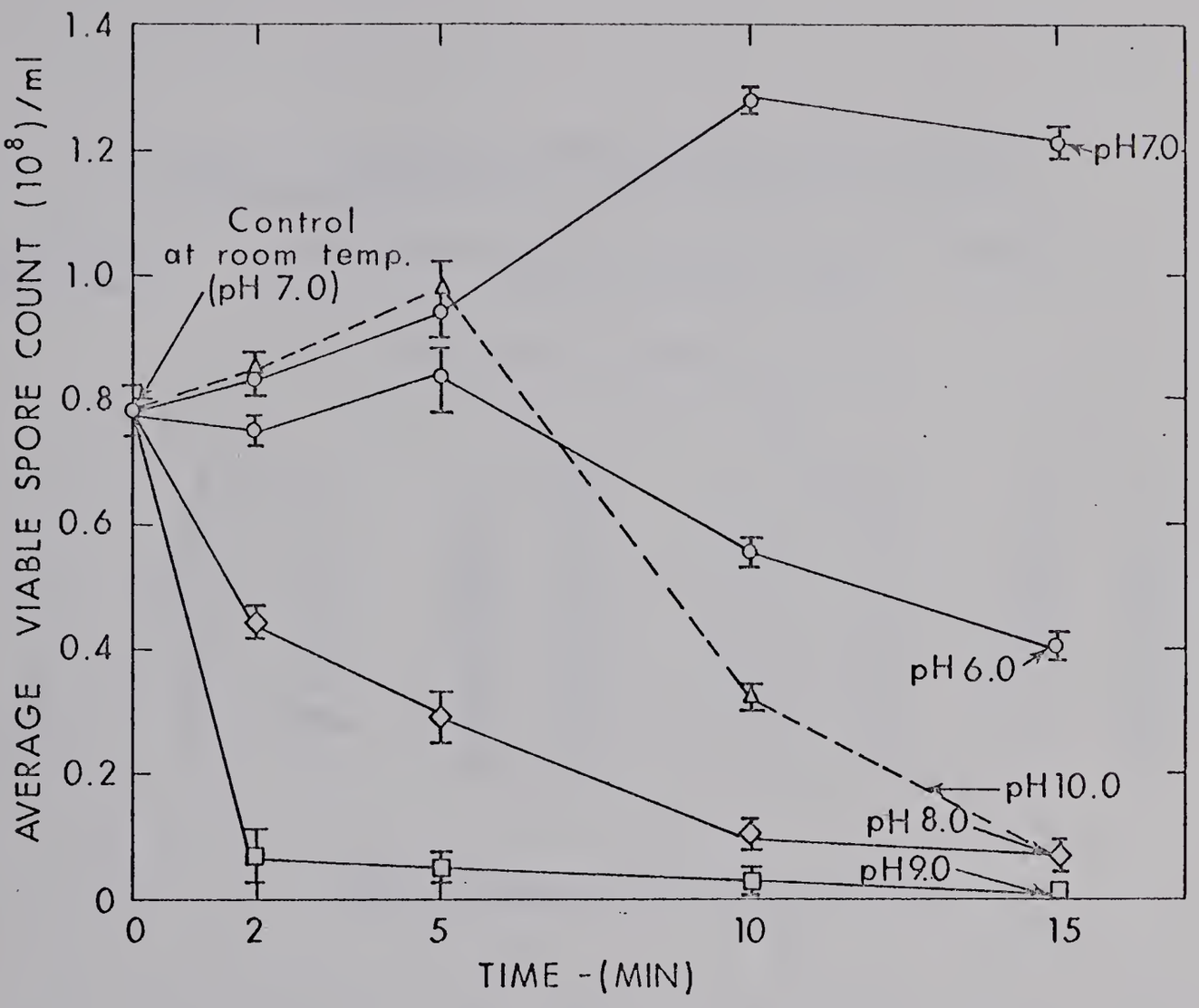

Fig. 13. The effect of pH and heat (95°C) on germination of spores of Bacillus subtilis 8057 at different exposure times.

Table 9. The effect of pH and heat (95°C) on germination of spores of Bacillus subtilis 8057 at different exposure times.

pH of Heating Menstruum	Average viable spore count (10^6)/ml and Standard Error of the Mean ($S\bar{x}$)							
	Time (min) of exposure at 95°C							
	2		5		10		15	
2	9	±0.7	4	±0.6	3	±0.6	0	± -
3	59	3.5	56	3.5	26	3.0	0	-
4	94	2.7	88	1.8	49	2.5	26	1.1
5	133	3.5	110	2.3	65	1.1	50	3.0
6	75	1.5	84	2.9	55	1.1	40	0.6
7	83	1.5	94	1.1	128	1.1	121	1.5
8	44	0.6	29	2.2	10	1.1	7	0.9
9	7	2.3	5	1.2	3	1.5	1	0.6
10	85	1.4	88	2.5	32	1.5	6	1.0
	Time of exposure at room temperature							
	2		5		10		15	
2	102	±1.0	91	±2.2	68	±0.6	63	±1.1
3	92	2.0	85	0.6	86	1.1	90	1.0
4	90	0.6	93	2.1	84	1.1	96	0.6
5	79	0.6	83	1.1	76	2.1	76	3.9
6	50	2.1	95	0.6	65	1.1	75	2.8
7	78	1.2	79	0.8	75	2.1	85	3.2
8	75	2.9	79	3.0	98	2.2	74	1.8
9	70	3.6	68	1.8	66	0.9	80	1.4
10	65	3.5	105	2.3	110	1.4	125	2.7
Control at pH 7.0 and 4°	79	1.8						

treatment times. Slight heat activation was permitted at short exposure times with pH treatments of 6.0 and 10.0.

The effect of pH without heat was also studied, and Table 9 gives the results. For reasons given in the discussion, the results are not presented graphically.

Keynan et al. (1964), working with Bacillus cereus T, found activation at 65°C for 45 min. From results of Fig. 8, heat treatments at 65°C gave significant activation between 20 and 60 min of treatment. Hence, in the study of the effect of pH plus heat on spores of B. cereus 127, a heat treatment of 65°C was chosen at four exposure times, namely 2, 5, 10, and 15 min. Fig. 14 and Table 10 summarize the results. Throughout the entire pH range from 2.0 to 10.0, no activation occurred, and most of the treatments were distinctly lethal. However, a double trend was observed over the pH range 2.0 - 10.0. At pH 2.0, 6.0 and 7.0, a marked lethal effect was evident with milder lethal effects at pH 3.0 and 8.0 - 10.0. It seems that the spores of Bacillus cereus 127 had no resistance whatever to the effects of pH and heat. The gradual reduction in viable spore count at pH 4.0 to 7.0 resembles the reduction trend that was termed "heat induced dormancy" by Finley (1964).

Table 11 summarizes the effect of pH alone on spores of B. cereus 127. Throughout the pH range, the four exposure times show viable spore counts consistently below that of the

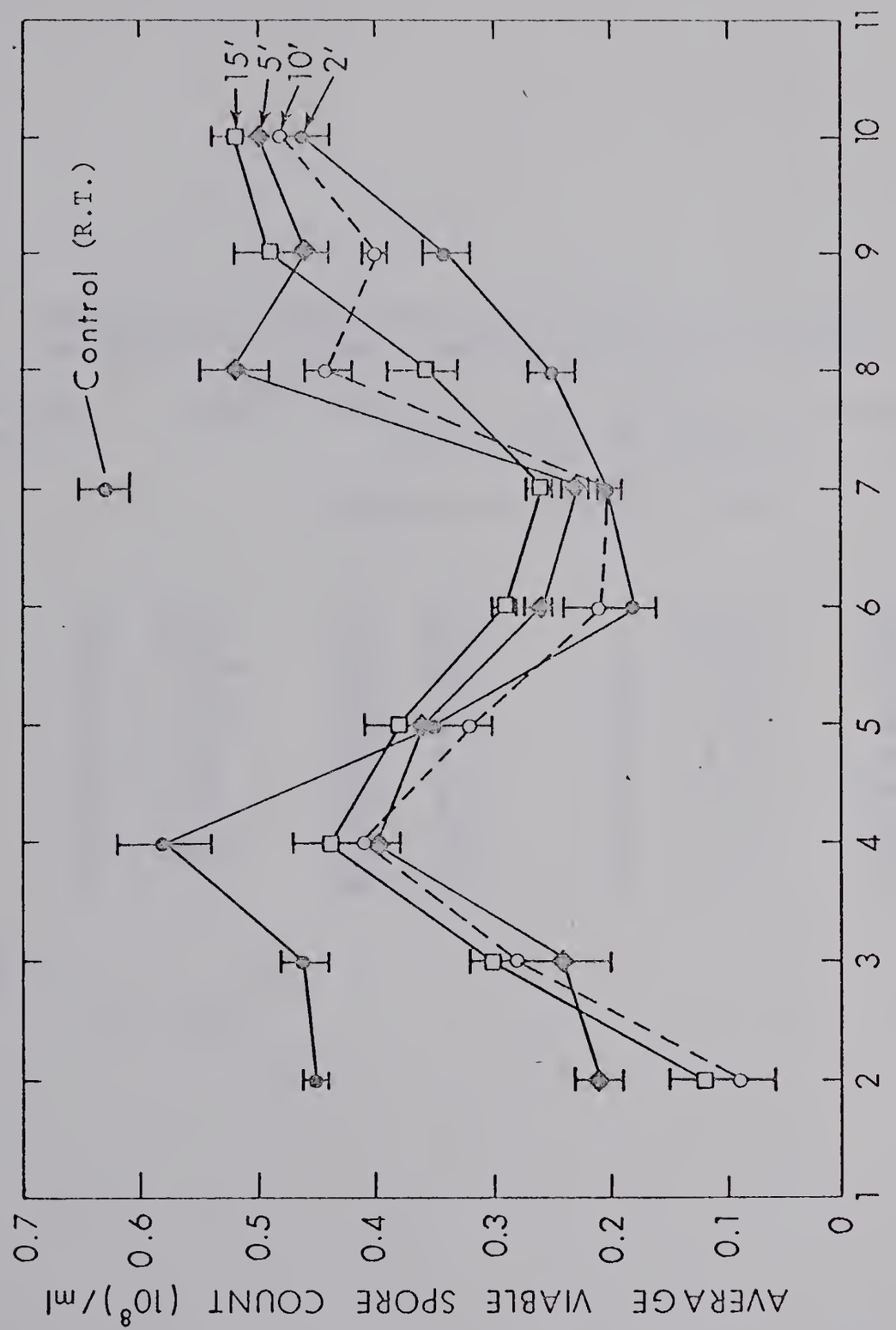

Fig. 14. The effect of pH and heat at different exposure times on germination of spores of Bacillus cereus 127 at 65°C.

Table 10. The effect of pH and heat at different exposure times on germination of spores of Bacillus cereus 127 at 65°C.

pH of Heating Menstruum	Average viable spore count (10^6)/ml and Standard Error of the Mean ($S\bar{x}$)							
	Time (min) of exposure at 65°C							
2	45	±3.2	21	±2.6	9	±2.3	12	±1.4
3	46	4.1	24	1.6	28	1.8	30	2.0
4	58	0.8	40	2.0	41	2.6	44	2.7
5	35	3.4	36	3.0	32	1.8	38	2.7
6	18	1.7	26	1.1	26	3.1	29	1.1
7	20	0.5	23	1.5	25	1.5	26	1.4
8	25	1.7	52	2.8	44	2.3	36	3.3
9	34	2.0	46	2.3	40	1.4	49	2.9
10	46	1.7	50	2.6	48	0.5	52	2.1
Control pH 7.0 and 4°	63	1.02						

Table 11. Effect of pH at different exposure times on germination of spores of Bacillus cereus 127.

pH of Menstruum	Average viable spore colony count (10^6)/ml and Standard Error of the Mean ($S\bar{x}$)							
	Time (min) of exposure at room temperature							
	2		5		10		15	
2	47	±4.7	42	±2.4	47	±1.1	31	±5.0
3	62	2.3	40	0.3	42	5.0	51	4.3
4	48	5.0	35	2.5	47	0.8	34	3.0
5	42	4.6	48	3.8	46	2.8	45	1.7
6	60	2.9	32	0.9	46	3.1	43	1.2
7	54	0.9	65	1.8	46	0.7	51	3.3
8	48	6.2	65	0.7	62	4.6	58	3.2
9	75	3.5	71	1.2	69	1.4	53	0.9
10	81	4.4	76	4.8	61	3.4	65	5.3

control. pH 10.0 at 2 min and 5 min shows viable spore counts that are significantly above the control, and statistically may be considered as activation, but this is very slight. Generally there is no significant difference in effect of pH at the four exposure times, and the effects, as seen from the results, are really lethal to the spores of B. cereus 127.

EFFECT OF THIOGLYCOLIC ACID ON ACTIVATION OF SPORES

Procedure

The reducing agent used was thioglycolic acid (TGA) in the concentration of 0.5%w/v, which had an initial reading of pH 6.4.

To determine the effect of TGA on spores of B. subtilis 8057, the free acid was titrated to pH 7.4 with a 6N NaOH, and added to the spores suspended in phosphate buffer. The acid spore suspension was incubated in a refrigerated water bath at 16 - 20°C continuously for 168 hr. At 24 hr intervals, 1 ml quantities were removed with a syringe, and introduced into ampules. The experimental plan was as follows:

(i) Reducing agent and heat - TGA spore suspensions were incubated for diffferent times and 1 ml quantities dispensed into sealed ampules which were heated in the oil bath for 10 min at 95°C.

(ii) Reducing agent alone - Method as given above (i)

(iii) Control

Plates were prepared to 10^{-6} dilution and incubated at 37°C for 24 hr.

Results

The effect of the reducing agent, TGA, on spores of B. subtilis 8057 was studied and the results are presented in Fig. 15 and Table 12. Spores were suspended in 0.5%w/v TGA and incubated at 16 - 20°C in a refrigerated water bath. Fig. 15 shows that after the spores are in 24 - 48 hr contact with TGA, followed by exposure to 95°C for 10 min, the viable spore count was below that of the control. Beyond 48 hr contact with TGA, the activation trend started upward, and at 72 hr, the viable spore count was significantly above the control. However, this activation was not of the order of that attained with heat treatment alone (Fig. 6). Between 72 and 96 hr contact the lethal effects on the spore commenced, and this trend continued rapidly up to 120 hr, after which it levelled off. All viable spore counts between 96 and 168 hr of contact, followed by exposure to heat, gave counts that were significantly below the control.

The effect of TGA alone was also compared to that of TGA and heat. Fig. 15 again shows no activation up to 24 hr of treatment. At 48 hr of contact with TGA, the activation trend commenced and this continued up to 72 hr of treatment. The viable spore counts which may have contained spores which germinated during treatment, at these times were significantly greater than that obtained for TGA and heat treatment.

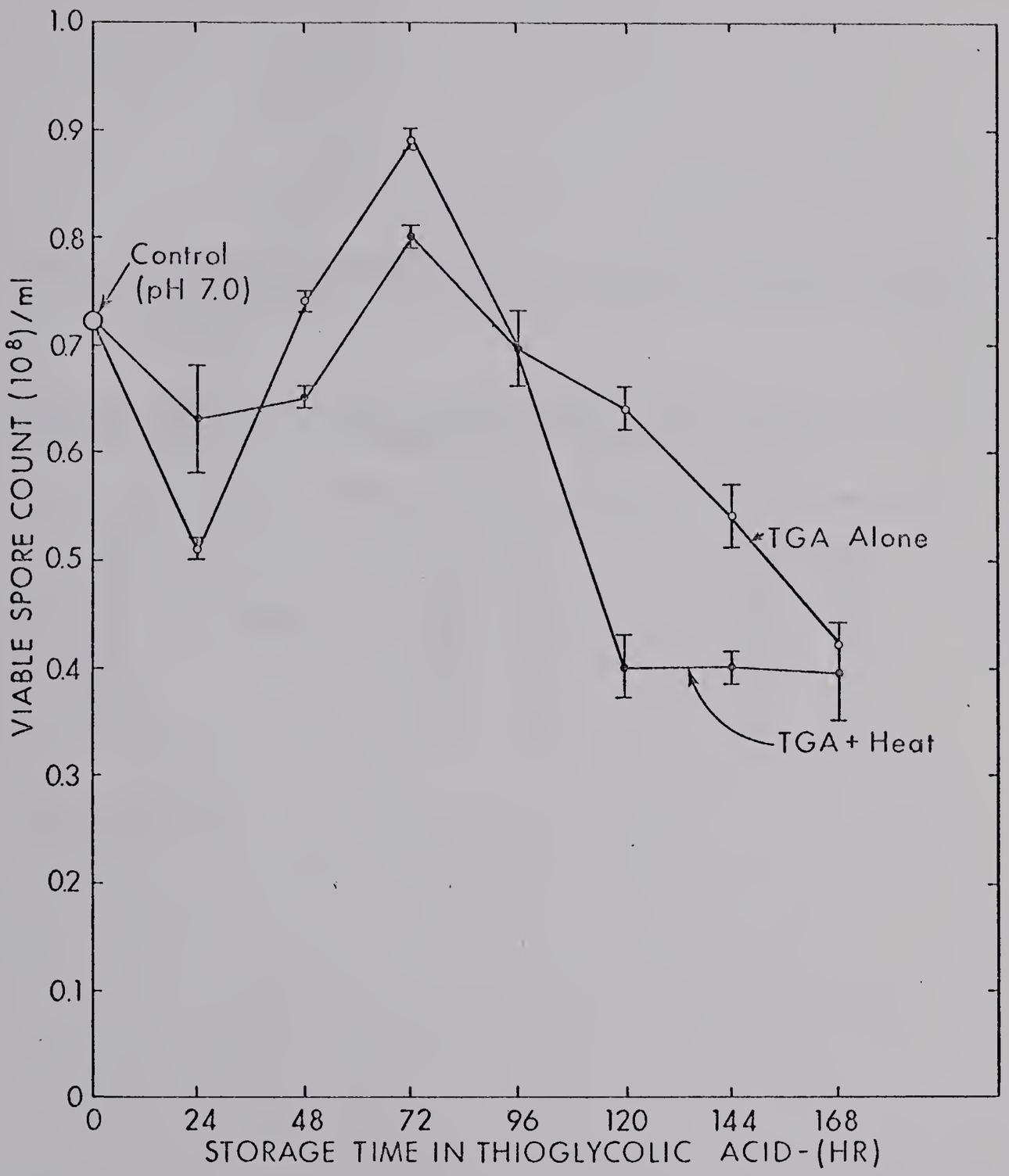

Fig. 15. Effects of thioglycolic acid and heat treatment at 95°C for 10 min on germination of spores of Bacillus subtilis 8057.

Table 12. Effects of thioglycolic acid and heat treatment at 95°C for 10 min on germination of spores of Bacillus subtilis 8057.

Storage Time in TGA (h)	Average viable spore count (10^6)/ml and Standard Error of the Mean ($S\bar{x}$) with:			
	TGA at 95°C for 10 min		TGA at room temp.	
24	63	±5.7	51	±1.0
48	65	1.1	74	0.6
72	80	1.1	89	1.3
96	69	1.0	69	3.8
120	40	3.2	64	2.3
144	40	1.5	54	3.5
168	39	3.9	42	2.3
Control pH 7.0 and 4°	72	1.1		

As the treatment time in TGA was increased, the lethal effects of TGA were seen. This lethal effect began immediately after 72 hr, and the trend was rapid up to 96 hr, followed by a more gradual but definite lethal action up to 168 hr. The counts between 96 and 168 hr of treatment were significantly below that of the control.

EFFECT OF CATIONS ON THE ACTIVATION OF SPORES

Procedure

To show the effect of cations on spore activation, reagent grade $CaCl_2$, $MnCl_2$, and $MgCl_2$ solutions of known purity were used.

Seven concentrations of each cation were prepared with concentration gradients of 1, 5, 10, 20, 30, 40, 50 x 10^{-4}M. These salts were dissolved in 25mM Tris buffer (hydroxymethyl amino) methane-Cl (pH 7.0), and was then made up to 100 ml quantities in screw cap bottles. After autoclaving at 121°C for 15 min, the mixture was cooled and stored at 5°C.

The method used was that of Splittstoesser and Farkas (1966). A typical activation trial consisted of pipetting 1 ml washed spores of a stock spore suspension into 9 ml of the prepared diluent, and equilibrating to room temperature by intermittant agitation with a Vortex mixer over a period of 2 min. Following this, the spores were allowed to remain in the respective cation solution for 10 min at room temperature.

To show the effect of heat and cations, 1 ml quantities of the spore-cation mixture were removed at 0 time, with a syringe, and introduced into ampules which were sealed and heated at 95°C for 10 min. The experimental plan comprised four parts:

(i) Heat alone - 1 ml stock spore suspension was introduced into ampules, sealed, and heated in the oil bath at 95°C for 10 min.

(ii) Respective cation and heat)
(iii) Cation alone)Method as given in (i).
(iv) Control)

Plates were prepared to 10^{-6} dilution and incubated at 37°C for 24 hr.

Results

$CaCl_2$ and Heat (95°C)

Fig. 16 and Table 13 summarize the results. They show that calcium in the presence of heat (95°C) has a lethal effect on the spores of B. subtilis 8057, regardless of the concentration of the cation in the heating menstruum. Concentrations 1×10^{-4}M to 50×10^{-4}M gave survival counts that were extremely low, and considerably below the control, the difference being significant throughout the entire range. At zero concentration, distinct activation is seen. Fig. 16 also shows the effect of $CaCl_2$ alone. It is seen that $CaCl_2$ produces activation throughout the concentration range, since all viable spore counts are above that of the control. However, only the concentrations between 1 and 20×10^{-4}M $CaCl_2$ gave significant activation. As the concentration of

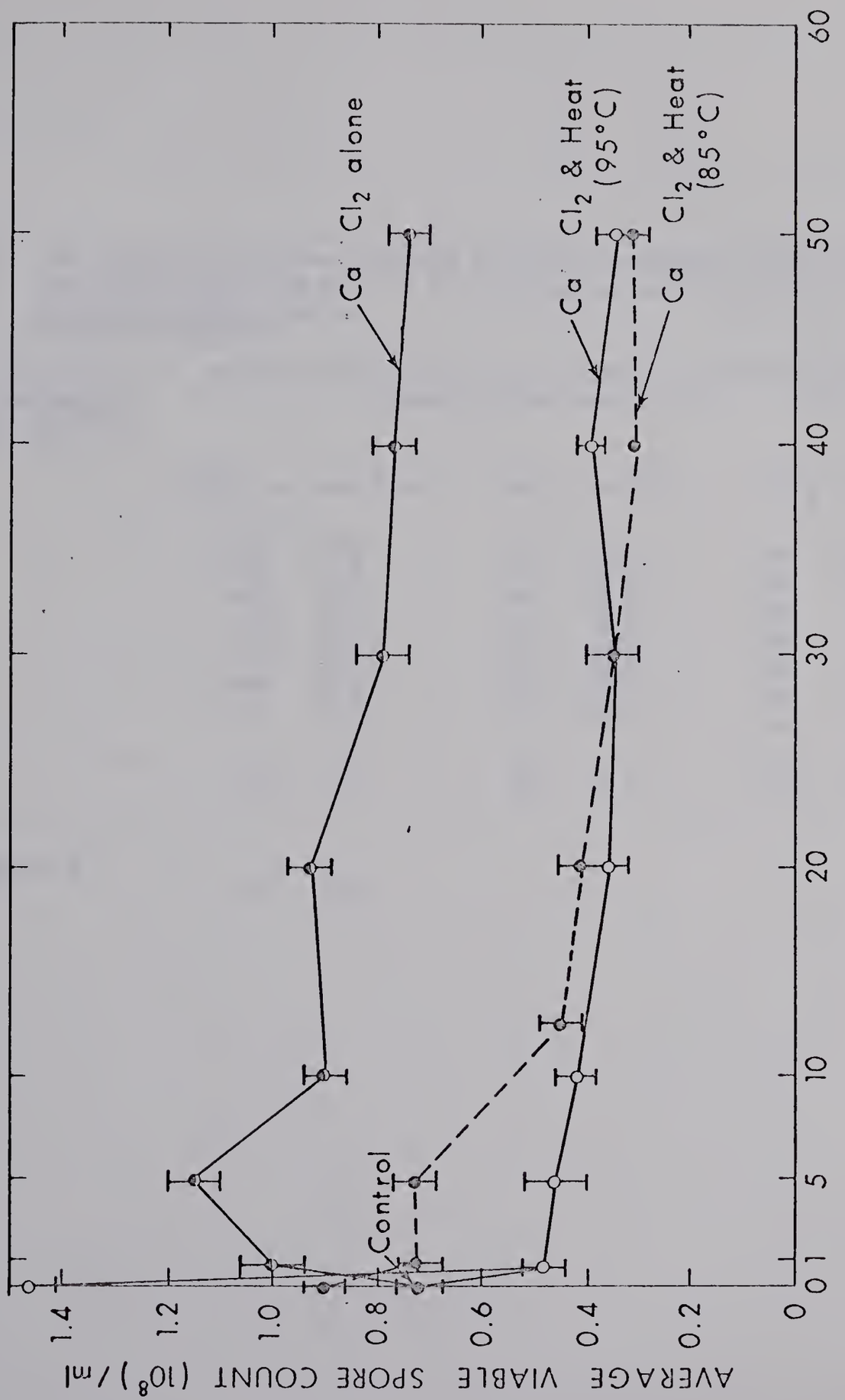

Fig. 16. The effect of calcium chloride and heat treatment at 85° and 95°C for 10 min (pH 7.0 - 7.1) on germination of spores of Bacillus subtilis 8057.

Table 13. The effect of calcium chloride and heat treatment at 85° and 95°C for 10 min (pH 7.0 - 7.1) on germination of spores of Bacillus subtilis 8057.

Concentration of $CaCl_2$ in Heating Menstruum (10^{-4} M)	Average viable spore count (10^6)/ml and Standard Error of the Mean ($S\bar{x}$)					
	$CaCl_2$ at room temp		$CaCl_2$ at 85°C		$CaCl_2$ at 95°C	
1	100	±2.0	72	±2.3	48	±2.3
5	115	1.8	73	2.0	46	3.0
10	90	2.5	45	1.8	42	2.4
20	93	2.1	41	1.7	36	1.7
30	79	1.9	35	2.1	35	2.0
40	75	2.5	30	0.0	39	0.9
50	72	1.7	31	1.1	34	1.8
0.0	72	2.1	90	1.9	146	2.8
Control pH 7.0 and 4°	72	2.07				

$CaCl_2$ increased from 30 to 50 x 10^{-4}M, viable spore counts are not significantly different from that of the control.

Since 95°C and $CaCl_2$ had a lethal effect on B. subtilis 8057 spores, it was considered interesting to see what effect 85°C would have. Fig. 16 shows that 1 x 10^{-4}M and 5 x 10^{-4}m concentrations gave counts comparable to the control. All other concentrations beyond 5 x 10^{-4}M gave viable spore colony counts that were similar to $CaCl_2$ and heat (95°C) treatment.

$MgCl_2$ and Heat

Fig. 17 and Table 14 show that heat alone (95°C) or zero concentration of the cation gave maximum activation. But all concentrations of $MgCl_2$ and heat (95°C) ranging from 1 x 10^{-4}M - 50 x 10^{-4} gave very low viable spore colony counts, indicating a distinct lethal effect of the spores, which was similar to the effects of $CaCl_2$ and heat.

Treatments of 85°C and the various $MgCl_2$ concentrations show that concentration 1 x 10^{-4} gave no activation, but a significant rise in viable spore count is observed between concentrations 1 x 10^{-4}M and 30 x 10^{-4}M. From concentrations 30 x 10^{-4}M to 50 x 10^{-4}M, a gradual drop in viable spore counts occurred.

The effect of $MgCl_2$ alone shows significant activation of the spores throughout the concentration range 1 x 10^{-4}M - 50 x 10^{-4}M with a peak occurring between 1 x 10^{-4}M and 20 x 10^{-4}M, beyond which there was a gradual drop. The viable spore counts in all

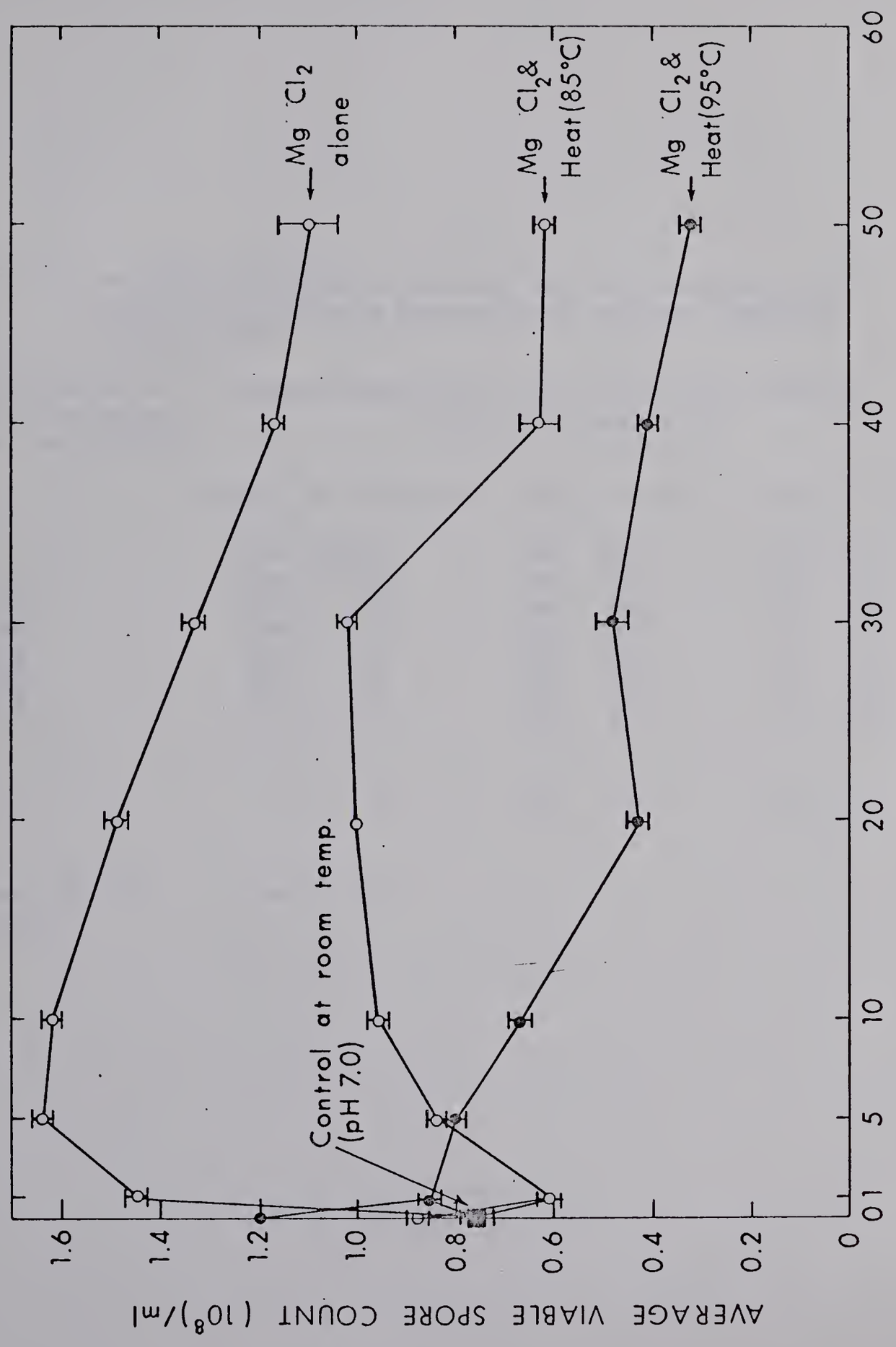

Fig. 17. The effect of magnesium chloride and heat treatment at 85° and 95°C for 10 min on germination of spores of Bacillus subtilis 8057.

Table 14. The effect of magnesium chloride and heat treatment at 85° and 95°C for 10 min on germination of spores of Bacillus subtilis 8057.

Concentration of $MgCl_2$ in Heating Menstruum (10^{-4}M)	Average viable spore count (10^6)/ml and Standard Error of the Mean ($S\bar{x}$)					
	$MgCl_2$ at room temp		$MgCl_2$ at 85°C		$MgCl_2$ at 95°C	
1	145	±0.6	61	±1.0	85	±0.6
5	164	0.7	84	0.6	80	0.6
10	162	0.6	96	1.1	67	0.9
20	149	1.5	100	0.5	43	1.1
30	133	1.1	102	1.5	48	1.5
40	127	1.5	63	2.1	41	1.0
50	110	3.0	62	0.6	32	0.9
0.0	76	1.5	88	1.3	120	1.5
Control pH 7.0 and 4°	76	1.5				

concentrations of $MgCl_2$ were statistically greater than all counts obtained from heat activation (Fig. 6).

$MnCl_2$ and Heat

The effect of manganese chloride is summarized in Fig. 18 and Table 15. They show that $MnCl_2$ and heat (95°C) was markedly lethal on the spores of B. subtilis 8057. This situation compares well with the other cations already mentioned and the viable spore counts throughout the concentration range 1×10^{-4}M to 50×10^{-4}M $MnCl_2$ showed a drastic lethal effect on the spores. The effects of $MnCl_2$ and heat at 85°C was next compared, and Fig. 18 again shows a distinct activating trend throughout the concentration range. Between concentrations 1×10^{-4}M and 10×10^{-4}M $MnCl_2$, the activation shown is significantly greater than that of the counts obtained with heat alone at 85°C. Beyond 10×10^{-4}M $MnCl_2$, up to 50×10^{-4}M, the viable spore counts showed no significant difference one from the other, although distinct activation is seen. The peak activation with $MnCl_2$ and heat (85°C) is between concentrations 1×10^{-4}M and 10×10^{-4}M.

The effect of $MnCl_2$ alone is also shown in Fig. 18, and again the viable spore counts obtained were statistically greater than the control viable spore counts. However, there

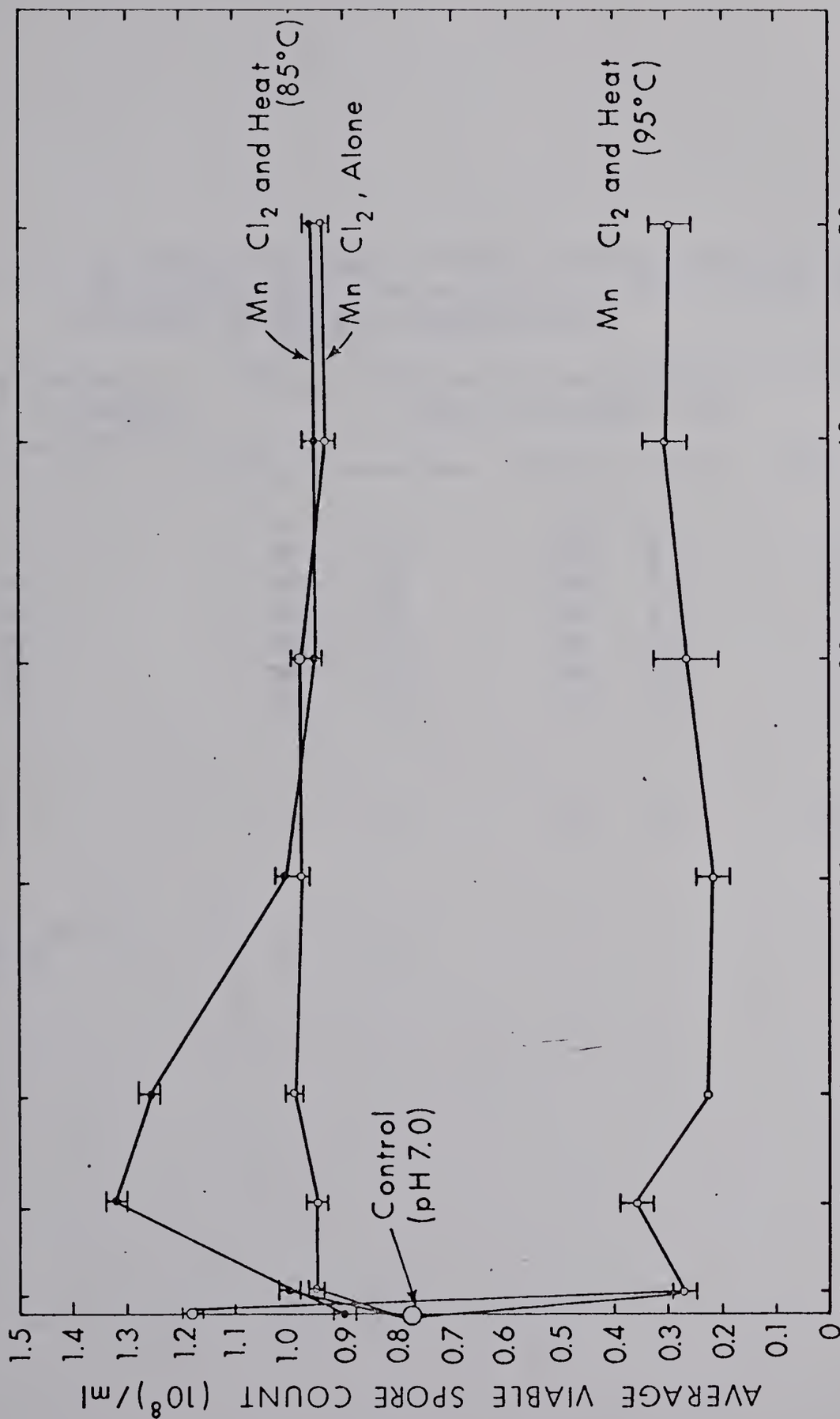

Fig. 18. The effect of manganese chloride and heat treatments at 85° and 95°C for 10 min at pH 7.0 - 7.1 on germination of spores of Bacillus subtilis 8057.

Table 15. The effect of manganese chloride and heat treatments at 85° and 95°C for 10 min at pH 7.0 - 7.1 on germination of spores of Bacillus subtilis 8057.

Concentration of $MnCl_2$ in Heating Menstruum (10^{-4}M)	Average viable spore count (10^6)/ml and Standard Error of the Mean ($S\bar{x}$)					
	$MnCl_2$ at room temp		$MnCl_2$ at 85°C		$MnCl_2$ at 95°C	
1	95	±1.5	100	±1.5	27	±1.6
5	95	1.5	132	1.7	36	3.2
10	99	1.1	126	1.6	23	0.3
20	98	0.6	101	1.8	22	3.5
30	98	1.1	96	2.3	27	6.0
40	94	1.1	96	2.3	31	4.0
50	93	1.7	97	1.5	30	4.0
0.0	77	1.7	90	1.6	118	2.0
Control pH 7.0 and 4°	77	1.7				

were no differences in result between concentrations of $MnCl_2$, in the range 1×10^{-4}M to 50×10^{-4}M, on the activation of spores. It is also shown that 1×10^{-4}M to 10×10^{-4}M concentrations of $MnCl_2$ and heat at 85°C gave peak activation, superior to the activation produced by $MnCl_2$ alone. However, these treatments show that with increasing concentration the viable spore counts indicate activation levels that were similar.

DISCUSSION

Keynan et al. (1965) suggest that activation is basically a change in, or a denaturation of, the tertiary structure of the protein responsible for dormancy within the spore, since activation inducers such as heat, low pH, and reducing agents (e.g. thioglycolic acid) are known to affect the tertiary structure of proteins. Furthermore, the dormant state is said to be brought about by the spore coat which is richly supplied in cystine moieties which are arranged in a specific configuration by S-S linkages. Reduction of these linkages would give a change in the tertiary structure which could cause activation, and conversely reoxidation of this bond would induce a reversion to the dormant state.

As has already been noted, the term 'activation' in this paper will be used to refer to treatments which subsequently lead to an increase in viable spore colony count.

Bacterial spores require some form of treatment before rapid germination will occur in a nutrient medium. Some workers found that spores which exhibit a greater heat activation tend to be more resistant to thermal destruction (Curran and Evans, 1945; Brachfeld, 1955; Finley, 1964). Thus it may be said that there is a relationship between the spores of thermotolerant and

and thermophilic organisms and their heat activation. Moreover, there may be some relationship between the heat resistance of spores and their specific gravity. Toda and Aiba (1966) noted that the specific gravity of spores is larger compared with the vegetative cells as a whole. In particular the specific gravity for the most heat-resistant bacterial spore of Bacillus stearothermophilus was found to be the largest in value among the cells studied. This may be ascribed to either a relatively poor water content or the higher content of nucleic acids in the spores (Lamanna et al. 1953).

The results obtained in the present investigation indicate that spores of B. subtilis 8057, which are mesophilic, do respond to heat activation. Activation, in differing degrees, occurs throughout the temperature range from 60° - 95°C at the varying time treatments. However, as Fig. 5 shows, the minimum time-temperature treatment for maximum spore activation of this organism is 95°C, for 10 min. It appears that this organism shows three definite behavior patterns. At 85°C, for 10 min, a peak of activation is attained, which may be termed a 'minor activation peak'. Apparently, this minor activation peak prepares the spore for the next behavioral pattern, with the increase in temperature. For, as is shown in Fig. 5, an increase in temperature, up to 90°C for 10 min, produces a significant reduction in subsequent viable spore colony counts. This behavior or

phenomenon has been reported by Finley and Fields (1962), working with Bacillus stearothermophilus, who termed it "heat-induced dormancy", and by Grecz (1967) who, working with radiation energy on spores of the genus Clostridium reported and termed this behavior "paradoxical inversion". Finley (1964) concluded that spores which succumbed to heat-induced dormancy were definitely more resistant to thermal destruction. This seems to be true in the case of B. subtilis 8057, because when the temperature treatment is increased to 95°C for 10 min, maximum activation occurred, but an increase of temperature between 95° and 115°C brought about a reduction in viable spore count. This reduction reached a critical level at 105°C, following which lethal destruction of the spores occurred, at 110 - 115°C.

No explanation has been advanced for this behavior of "heat-induced dormancy". Joly (1965) described denaturation of proteins as any modification of the secondary, tertiary, or quaternary structure of the protein molecule, excluding any breaking of the covalent bonds. In this definition suggested by Joly (1965), the meaning of secondary, tertiary, and quaternary structure is that proposed by Bernal (1958), and in view of this proposal, protein denaturation of this nature is better described as transconformation, rather than denaturation. Thus, to continue the observation of "heat-induced dormancy" as has been observed in spores of B. subtilis 8057, it is suggested that 85°C is just sufficient heat to produce transconformation of the spore

protein, so that a minor level of sub-lethal heat activation occurs, but the spore protein structure in its sublethal activated state retains a sufficient level of its specific configuration. As heat treatment is increased to 90°C, a critical level is attained, which may be specific for some biological system or mechanism within the spore structure, and which causes the transconformed protein to revert to the native state.

Lumry and Eyring (1954) stated that the secondary structure appears to be less easily reversed than the tertiary structure. Thus, it is further suggested that 90°C induces a reversion of the spore to the dormant state, but there may be some irreversible and reversible moieties in the spore protein which predispose it, so that increased heating at 95°C is just enough to provide additional sublethal treatment, resulting in maximum activation of the spores.

It is possible to surmise that the three behavioral patterns described above occur in the heating menstruum when the critical temperature range is attained, and each will become evident, depending on the time-temperature treatment at which the experiment is stopped. The fact that the lethal effects are observed above 95°C suggests that the activation produced at 95°C predisposes the spores to the irreversible state, so that delicate systems within the spore cortex become vulnerable to increased heat from increasing temperatures, and accordingly the spores are destroyed.

Most workers assumed that heat activation was a convenient but artificial way of initiating the physiological 'aging' process in the laboratory. Other sources of protein undergo modifications during aging in solutions, and this can be classified as typical denaturation. Moore and Mayer (1944) reported a slow component appearing from an originally homogenous sample of horse serum albumin within two months at room temperature, and that the same phenomenon occurred within a few hours at higher temperatures. Keynan et al. (1964) further reported that the phenomenon of aging is non-reversible. Thus, this argument may be assumed to be the cause of the lethal effects of spores at 105 - 115°C, following the maximum heat activation which occurs at 95°C.

Spores of B. cereus 127, although mesophilic, as are those of B. subtilis 8057, do not respond spectacularly to heat activation. If one should accept the view of Finley (1964) that spores which exhibit greater heat activation tended to be more resistant to thermal destruction, then as Fig. 7 shows, B. cereus 127 spores exposed to treatments immediately beyond 85 - 90°C for 10 min suffered lethal effects. Thus, it may be said that B. cereus 127 spores, being of lower thermal resistance, show no dramatic activation when exposed to sub-lethal heat treatments. The temperature of 65°C for 10 min produced very mild activation, since the viable spore count was equal to that of the control.

Keynan et al. (1964), working with spores of B. cereus T, reported optimal germination rates after heat activation of 65°C for 45 min. When spores of B. cereus 127 were exposed to treatment at 65°C with increasing time, effective activation was observed (see Fig. 8), only between 20 and 60 min. It is assumed that this organism is genetically different from B. subtilis 8057, and accordingly it may possess biological systems that succumb to comparatively low heat treatment. It might be useful to do a detailed biochemical analysis of the protein components that comprise both organisms to see if there are quantitative and qualitative differences, which might be responsible for their different behavior to heat.

It is generally recognized that both the nature and the concentration of ions play a very important part in the denaturation process, and pH is particularly determinative, since almost all proteins are affected by sufficient concentrations of H^+ and OH^- ions. Globular proteins may be divided into classes according to their response to extremes in pH: those which apparently retain their structural integrity, those which expand, and those which dissociate. Some proteins are rapidly denatured at pH levels not far removed from the iso-ionic point, while others appear to react reversibly with acid and base over a large pH range. The particular behavior varies with the protein species, even if they belong to the same type of protein molecules (Joly,1965).

Spores of B. subtilis 8057 responded to pH in different ways, depending on the range of the pH and exposure times, as may be seen in Table 7. A graph of these results gave no clear-cut picture and is not presented with the results. The confusing picture was probably because the conditions allowed spores to germinate and the vegetative cells to survive for part of the experimental treatment, if not even to some small degree to multiply. It would appear that with experiments involving heat with its lethal or sub-lethal effect that we are only concerned with spores. However, with treatments not involving heat but carried on over a period of time some germination would seem not unreasonable, resulting in the experimental treatments acting against spores and vegetative cells simultaneously yielding a situation so complex that they are not subject to analysis.

Gibbs (1967), working with anaerobic Clostridium bifermentans, spores, observed successful activation responses at pH 2.0 and at pH 10.0 and above. When spores of B. subtilis 8057 were exposed to pH and heat (at temperatures ranging from 65 - 95°C for 10 min) two activation points were noted (See Figs. 9 and 10). pH 8.0 at 70°C gave maximum activation as did pH 7.0 at 95°C. At pH 8.0, and 70°C, the activation produced resulted from the combined effects of pH and heat, while at pH 7.0 and 95°C, activation resulted from the effect of heat alone. At all temperatures pH 2.0 and 3.0 produced lethal effect on the spores.

It is possible that highly acid or highly alkaline (Figs. 12, 13) pH activates the spores, and when this occurred in the presence of heat, the combined effect was sufficient to be fatal to the spores.

A question arising from the experimental results of pH and heat for 10 min on the spores of B. subtilis 8057, was whether changes in time of exposure would have any profound effect on the responses of the spores. Exposing the spores to pH 6.0 - 8.0 at 70°C for 2, 5, 10, 15 min (Fig. 11) resulted in activation which was greatest at pH 8.0 at 70°C for 10 min. When the intensity of heat treatment was increased, activation changed to destruction. Spores exposed to 95°C and pH 5.0 for 2 min produced maximum activation. When the time of exposure to this pH and heat (95°C) was increased, the effect was decidedly lethal (Fig. 12). Again when the pH was made more acid (e.g. pH 2.0 - 3.0) and heat at 95°C applied regardless of the duration time, the effect was markedly lethal, and became more pronounced with increasing time. Lewis et al. (1965) found a comparable situation with spores of B. stearothermophilus. They reported that the spores treated in acid were heat labile, with no survivors after heating for 4 min at 115.6°C. When the pH was increased to 6.0 and heat at 95°C applied, the viable spore count was reduced, indicating that some reversion to the dormant state was taking place. At pH 7.0 and heat (95°C), activation was clearly

demonstrated, but this effect was clearly the result of heat alone. B. subtilis 8057 spores in alkaline pH and high temperature (95°C) were not activated at all; the response was similar to that of the low pH range, and Fig. 13 shows a distinct lethal effect.

At a certain pH value and heat treatment, activation of B. subtilis 8057 is induced which mimics the activation caused by heat alone but which is at a different level of heat treatment. It is clearly shown that acid pH (e.g. pH 5.0) will produce maximum activation at high temperatures (95°C) and short times (2 min), while alkaline pH and high temperatures are lethal regardless of the exposure times. Conversely, a lower temperature (70°C) requires an alkaline pH (pH 8.0) and longer exposure time (10 min) to produce maximum activation.

Spores of B. cereus 127 did not show any activating response when exposed to a change in pH alone, pH and heat, or heat alone. With pH alone, varying exposure times did not show any differences in the treatment (Table 11). The responses to acid pH and alkaline pH were similar, and the effects were consistently lethal. With heat treatment at 65°C, distinct thermal destruction of spores was effected (Fig. 14). It appears that under the influence of pH and heat there is a transitional stage between pH 5.0 and 8.0 where spores mimicked the reversion to dormancy, as was seen with B. subtilis 8057 under heat treatment

alone. In the case of B. cereus 127 spores, this behavior resulted from the combined effect of pH and heat (65°C); neither heat nor pH alone showed this effect; here again exposure times made no difference. This reversion to dormancy is tantamount to reversible and irreversible denaturation which occur simultaneously or successively. This may be the result of several factors such as changes in the pressure at the time of the experiment, cooling to room temperature, or possibly the phenomenon of competition which occurs when two denaturing forces are applied at the same time, as in the case of pH and heat in this experiment.

Joly (1965) has reported that in some cases the simultaneous action of two or more denaturing agents results in an effect smaller than that produced by one only. The protein behaves as if the denaturing processes controlled by each of the denaturing agents were incompatible, resulting in mutual inhibition. Perhaps this may have resulted in the behavior of B. cereus 127 spores between the pH 5.0 and 8.0, when exposed to heat at 65°C.

Spores of B. cereus 127 showed an increase in colony count between pH 8.0 and 10.0 with heat (see Fig. 14), and this behavior resembled that change from dormancy to activation exemplified by spores of thermotolerant organisms, such as B. subtilis 8057 (Fig. 5), and B. stearothermophilus already discussed. However, with spores of B. cereus 127, the apparent rise in count in this pH range (Fig. 14) was always below the control, so that the sum

total of the effects was lethal to the spores.

According to Keynan et al. (1964), certain chemical agents could mimic heat activation. Spores of B. subtilis 8057 were activated with thioglycolic acid and even though the level of activation achieved was significant (Fig. 15), this level of activation was not as marked as that of heat activation (Fig. 6). Thus, even though thioglycolic acid mimics heat activation, it does not produce as spectacular results as heat activation. Keynan et al. (1964), working with B. cereus T, further observed that a minimum time of pre-incubation of the spores with the reducing agent was required since no effect was observed in less than 12 hr. The spores of B. subtilis 8057 seemed also to require a minimum time of pre-incubation before activation took place, and Fig. 15 shows that between 48 and 72 hr was the minimum pre-incubation time required for activation.

While thioglycolic acid and heat (95°C - 10 min) produced obvious activation, the present studies have shown that a better response is obtained when the spores are pre-incubated in thioglycolic acid alone for 72 hr. Lindley (1955) reported that insulin pre-treated with urea, denatured the protein which when further treated with thiols such as thioglycolic acid, resulted in complete reduction of the disulfide bonds. According to Vinter (1960), spore coat protein is rich in cystine disulfide bonds. In this way, permeability or exposure to enzymes is increased, thus facilitating activation

of the spores. Spores of B. subtilis 8057 in the present work apparently respond favorably to thioglycolic acid, suggestive that rupture of the protein disulfide bonds takes place in the spore coat.

Although several workers have reported that ions enhance or inhibit spore germination (Foerster and Foster, 1966; Jaye and Ordal, 1965; Murrell, 1961; Rode and Foster, 1962, 1966) only very few reports have been made on the effects of ions on activation. Dormant B. stearothermophilus spores were activated by hydrogen ions (Levinson and Sevag, 1953) and Bacillus megaterium spores were activated by Ca^{++}, Sr^{++}, and Ba^{++}.

The findings from the present study of the effects of cations (Ca^{++}, Mg^{++}, and Mn^{++}) on the activation of Bacillus subtilis 8057 spores suggest that divalent cation treatment has a stimulatory effect on activation. Divalent cations combined with heat treatment (85° and 95°C) may be activating, or deactivating, depending on the respective cation-heat treatment involved.

Halmann and Keynan (1962) have reported that $CaCl_2$ (6×10^{-4}M) reduced by 50% the decrease in OD of a spore suspension of Bacillus licheniformis when added before or together with L-alanine after 10 min incubation at 37°C. Ca^{++} on the other hand, inhibited the "trigger" reaction, but not subsequent germination of B. licheniformis spores. The results of the present study show that 1×10^{-4}M to 20×10^{-4}M concentration of $CaCl_2$ produces significant

activation (i.e. increase in the subsequent viable spore count) of B. subtilis 8057.

Calcium ions are well known for their property of stimulating or accelerating protein denaturation in vitro. According to Halmann and Keynan (1962), in their work with Bacillus licheniformis, the addition of $CaCl_2$ to a spore medium inhibits activation; apparently salts are adsorbed on the spores, and this imparts some protection against heat during the heating process. However, in the present studies on spores of B. subtilis 8057, the results showed that $CaCl_2$ alone activates the spores, although the concentration (5×10^{-4}M) is similar to that of Halmann and Keynan (1962). This rules out the possibility of $CaCl_2$ having a general protective effect on the spores. The fact that $CaCl_2$ enhances heat denaturation of proteins in vitro does not discount the above findings, because the states of $CaCl_2$ within the spore, Ca^{++} present in the heating menstruum, and heating the spores with calcium, are three distinct situations that should not be similarly compared.

According to Keynan et al. (1965), activation is basically a denaturation of tertiary structures of proteins within the spore. If spore activation due to $CaCl_2$ is due to denaturation of protein components within the spore, this is in agreement with the generally accepted properties of proteins in vitro. But this statement cannot be overemphasized because the conditions of the reactions between $CaCl_2$ and spore protein in vivo and in vitro are decidedly

different. In vivo, there is a permeability barrier between the spore protein and $CaCl_2$ whereas in vitro there is a direct contact between $CaCl_2$ and the spore protein.

Lewis et al. (1965) suggested that the Ca^{++} content of spores might be correlated with their degree of dormancy but this does not rule out the validity of the present findings, because in Lewis' suggestion, Ca^{++} is chelated with diaminopimelic acid (DPA), forming a complex arrangement within the cortex protein of spores.

Heat alone produces significant activation, also $CaCl_2$ alone in concentrations of 1×10^{-4}M - 20×10^{-4}M produces comparable activation, but a combination of the high temperature (95°C) and $CaCl_2$ results in a definite destruction of the spores of B. subtilis 8057.

Joly (1965) has reported that the most common association of denaturing treatments is that of heating combined with other methods of denaturation, which generally leads to an augmentation of the denaturation effect. Vujicic, Batra and DeMan (1967) reported that calcium and magnesium ions were similar in magnitude for the removal of sodium as well as hydrogen ions, which led to the formation of complexes with polyphosphates in casein. It is possible therefore that calcium along with other cations studied in the presence of heat, becomes complexed with some exposed component in the spore protein, most probably phosphate moieties,

and thus the additive effect of the two denaturants results in the lethal destruction of the spores.

Among the three cations studied, it is clearly seen that magnesium gives the best activation of spores of B. subtilis 8057. Joly (1965) states that the action of various salts is comparable to denaturation, for example the molecular weight of serum globulin increases in 1M- ammonium sulfate. Again, lyotropic salt solutions cause denaturation, and Venkataraman (1960) attributes the action of these salts to the solvation effect. Thus, it is possible that magnesium has greater affinity for the spore protein, which leads to denaturation and consequently to maximum activation. But, when spores suspended in concentrations ranging from the very weak up to the potent level, were exposed to high temperatures, they showed a reverse effect, that is, very low viable spore counts. This indicates that $MgCl_2$ and heat (95°C) was extremely lethal. It is further seen that $MgCl_2$ at lower concentrations and 95°C was more lethal than at the lower temperature (85°C).

Probably Mg^{++} initiates some of the biochemical reactions fundamental to the activating process more readily than does heat, and the results of these studies clearly show that $MgCl_2$ especially in increasing concentrations is by far a superior sub-lethal agent for spores of Bacillus subtilis 8057. Manganese on the other hand, gives activation but not to any spectacular extent. However manganese in combination with high temperatures (95°C) elicits marked

lethal effects on the spores, as compared to the other cations and heat.

The general behavior of Mg^{++} and Mn^{++} is not different from that of Ca^{++} and it is possible that there is a common mechanism for divalent cations which produces a common effect on spores of B. subtilis 8057. Splittstoesser and Farkas (1966), working with Bacillus popilliae spores, stated that divalent cations may be required for activation since ethylenediaminetetraacetic acid inhibited activation, and potassium was shown to be competing with calcium for some active site.

The present work shows that B. subtilis 8057 in some degree or other responded to all the individual agents to which they were exposed; heat, pH, reducing agents, and cations. Intensifying the application of most of the agents used had a lethal rather than an activating effect. Where two agents simultaneously were used, the effect seemed to be synergistic and in many cases similar to the excessive application of an activating agent. With pH, however, one cannot consider alteration as an intensification, and therefore, at the extremes of the pH range used the spores exhibited activation but probably for different reasons.

With B. cereus 127, a less heat resistant organism, less activation and more lethal effect was exhibited as a result

of all the experimental treatments.

As far as possible practical applications are concerned from the above findings, the most marked activating effect resulted from certain sub-lethal time-temperature treatments; this is no new information.

However, the combined effect of moderate heat (95°C) and pH 2.0 was markedly more lethal than the application of heat alone. This may be in part why high acid foods require less heat treatment than low acid foods, and suggests a possible acid treatment of certain foods prior to a reduced heat treatment followed by aseptic neutralization if necessary. Again, a judicious application of cations even in minute quantities might be used because of their highly lethal effects on spores, in the presence of heat, e.g. in meat processing.

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